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STUDIES ON SALT AND WATER BALANCE IN CADDIS LARVAE (TRICHOPTERA):

I. OSMOTIC AND IONIC REGULATION OF BODY FLUIDS IN *LIMNEPHILUS AFFINIS* CURTIS

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I. INTRODUCTION

The present series of investigations on caddis larvae was begun in order to examine the regulatory mechanisms concerned with salt and water balance under conditions of relatively high external salt concentrations. The only previous investigation of salt regulation in caddis larvae is that of Boné & Koch (1942), who studied the regulation of chloride in the haemolymph of *Limnephilus flavicornis* larvae kept at very low external chloride concentrations.

The regulatory abilities of several other aquatic insects kept in saline media are known in some detail. In particular, salt and water regulation in *Aëdes aegypti* larvae has been studied intensively by Wigglesworth (1933, 1938) and Ramsay (1950, 1951, 1953), followed by tracer studies on sodium balance (see Stobbart, 1960). Larvae of *Sialis lutaria* were also thoroughly investigated by Beadle & Shaw (1950) and Shaw (1955*a*, *b*). In both *A. aegypti* and *Sialis* larvae the haemolymph osmotic pressure rises when the external salt concentration is increased, so that the haemolymph remains hyper-osmotic to the medium. Regulation of the haemolymph salt concentration begins to break down when the external salt concentration is increased to a level roughly equivalent to the normal total concentration of the haemolymph, i.e. at about 170 mM./l. NaCl. Larvae begin to die when the external concentration is increased above this level. External concentrations greater than about 170 mM./l. are also fatal to larvae of *Corethra* (Schaller, 1949) and *Helodes* (Treherne, 1954*a*). Thus, to all of these freshwater insects, external concentrations greater than 30–40% sea water are rapidly fatal. In distinct contrast, larvae of *Aëdes detritus* tolerate external salt concentrations more than twice as great as that of normal sea water. Furthermore, the haemolymph remains markedly hypo-osmotic to these very high salinities (Beadle, 1939). The larvae of several other aquatic Diptera also possess remarkable powers of hypo-osmotic regulation in very high salinities (Nemenz, 1960; Sutcliffe, 1960*a*).

It appears, then, that the above aquatic insects fall into two sharply contrasting groups with regard to salt tolerance and osmoregulation. It is therefore of interest to examine the regulatory abilities of an insect which tolerates a salinity range intermediate between these two groups. Larvae of *Limnephilus affinis* have long been known to occur in both fresh- and brackish-water habitats in north-west Europe (Silfvenius, 1906; Marlier, 1949). In Britain, *L. affinis* larvae were found recently in fresh water

immediately above an estuary and in nearby salt-marsh pools on the coast of Northumberland (Sutcliffe, 1960*b*). Sutcliffe reared imagines of *L. affinis* from larvae kept in 50% sea water, and found that larvae survive for long periods in 75% sea water. Moreover, in the salt-marsh pools larvae are periodically subjected to nearly full-strength sea water for several days at a time (Sutcliffe, 1961*a*) This paper describes salt and water regulation in the body fluids of *L. affinis* larvae kept at external salt concentrations ranging from < 1 to 470 mM./l. NaCl. Quantitative estimations of salt-water intake and output in *L. affinis* larvae will be given in a later paper.

II. METHODS

L. affinis larvae were obtained from pools in a salt marsh at Seaton Sluice, Northumberland. Larvae were kept in tanks of dilute sea water in the laboratory and were fed on dead sycamore and elm leaves until required for investigation. Larvae were not fed during the course of experiments, and they were removed from the portable case which these larvae possess. Larvae were kept individually in 3 × 1 in. tubes containing about 15 ml. of the experimental medium. The tubes were maintained at a little below room temperature in a tank through which tap water flowed continuously. Although the temperture of the water fluctuated during the year, it remained constant to within ±2° C. during the course of an experiment. Most of the experiments were carried out at 14–17° C.

Media were Newcastle tap water and local sea water from Cullercoats diluted to the required concentration with tap water. The tubes were normally filled with fresh media every 48 hr. Throughout this paper the salt concentrations of sea-water media are referred to in terms of equivalent concentrations of sodium chloride (mM./l. NaCl) and, for convenience, sea-water media are referred to as solutions of sodium chloride. No experiments were carried out with sodium chloride solutions in place of diluted sea water.

Body weights were determined to the nearest 0·5 mg. on a 500 mg. torsion balance. The wet weight of large, mature larvae was about 40–50 mg. Larvae with body weights of less than about 25 mg. were not normally used for experiments.

Body fluids were usually analysed only from larvae kept for 6–10 days at the final experimental salt concentration. It was possible to transfer larvae directly from tap water into 410 mM./l. NaCl without harm, but the normal procedure was to increase the external salt concentration by stages of about 100 mM./l. NaCl, at intervals of 2–3 days, to the required concentration.

Haemolymph samples were withdrawn from a small puncture made in the mid-dorsal region of the anterior abdominal segments. Larvae were first narcotized with CO_2 bubbled through the experimental medium, washed rapidly with a jet of distilled water, and dried with filter-paper. Samples were stored temporarily under liquid paraffin in Silicone-lined watch-glasses. Larvae were not used again after removal of haemolymph.

Rectal fluid was collected with a fine glass pipette, employing the technique used on *Sialis* larvae by Shaw (1955*b*). Larvae were not narcotized for this operation as it was found that in many cases the rectal fluid was discharged during treatment with CO_2. Instead, larvae were gripped and held in the abdominal region with blunt forceps.

As in *Sialis* larvae, rectal fluid was often discharged on touching the tip of the abdomen with the pipette. When fluid could not be obtained in this way the tip of the pipette was eased gently past the anal sphincter and into the rectum. The volume of fluid obtained varied with the concentration of the external medium. From larvae kept in tap water it was relatively easy to obtain 1–2 μl. of rectal fluid in a single, daily collection. At external concentrations above about 200 mM./l. NaCl, quantities greater than 1 μl. were not often obtained.

The rectal fluid was normally colourless, but occasionally varied in colour from pale straw to dark brown. This was presumably due to contamination with the dark brown midgut fluid. All discoloured samples of rectal fluid were discarded.

Osmotic pressures of body fluids and sea-water media were determined with the micro-cryoscopic technique of Ramsay & Brown (1955). Temperatures were read to the nearest 0·005° C. and readings on the same sample were reproducible to $\pm$0·005° C. Osmotic pressures are expressed in terms of NaCl solutions with equivalent freezing-point depressions. For concentrations up to 513 mM./l. the relationship between concentration and the freezing-point depression of NaCl solutions was reasonably linear, and the value of Δ/171 mM./l. NaCl = 0·610° C. was found empirically. This is slightly higher than the value of 0·60° C. obtained by Ramsay (1949). The osmotic pressure of body fluids was occasionally not determined until 24 hr. after removal from larvae. During the intervening period samples were stored under liquid paraffin in Pyrex glass capillaries at approximately $-8°$ C. The osmotic pressure was not significantly altered by this treatment.

Conductivity. The conductance of small samples (about 1 μl.) was measured in a Perspex cell of the type described by Shaw & Staddon (1958). Samples were introduced quantitatively into the cell filled with a standard volume (about 120 μl.) of de-ionized water. The cell was connected to a Mullard conductivity bridge. Both were calibrated empirically against NaCl solutions introduced with the sample pipette. Duplicate readings were made whenever possible, accurate to $\pm$3 mM./l. NaCl.

Chloride. Measured by the ultra-micro Volhard titration devised by Wigglesworth (1937). Measurements were made in duplicate or triplicate, accurate to $\pm$3 mM./l. chloride.

Sodium. Estimated with an EEL Flame Photometer. Small samples (2–3 μl.) were delivered quantitatively into 2 or 3 ml. de-ionized water in small polythene cups. During each series of measurements the flame photometer was calibrated empirically with standard NaCl solutions prepared in the same manner. Whenever possible duplicate estimations were made on each sample, accurate to $\pm$3 mM./l. sodium.

Labelled sodium. Permeability of the body wall to sodium ions was investigated by measuring ^{24}Na influx from sea-water media. The results were also used to estimate the quantity of water imbibed through the mouth, and it was necessary to prevent any undue disturbance of the larvae. Rectangular boxes were constructed with Perspex and each box was partitioned inside with vertical strips of celluloid to form twenty-four small compartments. These were not completely isolated from one another as the celluloid strips did not quite reach the bottom of the box. This arrangement prevented larvae from interfering with each other but allowed some movement of the medium between compartments. Each box was filled with 200 ml. experimental medium and one larva was placed in each compartment. After 5–6 days acclimatization

one half of the larvae were prevented from drinking the medium by sealing the mouth with a small blob of 'Sira' wax. They were then returned to the medium for a further 2 days. The medium was then gently tipped out and fresh medium (filtered) of the same concentration containing a very small quantity of labelled sodium was run in with a syringe pipette.

Larvae were removed from the radioactive media at intervals over 100 hr. and the radioactivity of a known volume of haemolymph (2–5 μl.) was assessed under an end-window Geiger counter in the usual way. After removal of haemolymph larvae were discarded from the experiment. During the first 70 hr. the counting error for individual samples was about $\pm 2\%$. Between 70 and 100 hr. the counting time was extended so that the counting error did not exceed $\pm 5\%$. In each experiment the standard deviation of counts on six to eight samples delivered from the same sample pipette was less than $\pm 3\%$. Appropriate corrections were made for background and decay. All counts are expressed as counts/μl./5 min.

The decay of ^{24}Na was measured over periods of up to 60 hr. On each occasion the half-life was 15·1 hr. There seems to be no general agreement on the actual decay rate of ^{24}Na (see Treherne, 1954*b*) but most of the recent estimates indicate that the half-life probably lies between 15·0 and 15·1 hr.

III. REGULATION OF THE HAEMOLYMPH OSMOTIC PRESSURE

The relationship between osmotic pressure of the haemolymph and that of the external medium closely resembles the relationship found in a number of freshwater animals. The mean haemolymph osmotic pressure of larvae kept in tap water was $129 \pm 14{\cdot}5$ mM./l. NaCl ($N = 17$). In sea-water media the haemolymph osmotic pressure increased to become iso-osmotic at an external concentration of about 200 mM./l. NaCl. At higher external concentrations the haemolymph osmotic pressure continued to rise and, although it remained roughly similar to that of the medium, some variation was found in the extent to which the osmotic pressure increased. For example, in one series of experiments groups of six to seven larvae were acclimatized to external concentrations ranging from tap water to 427 mM./l. NaCl. Haemolymph osmotic pressure, conductivity and sodium concentration in each larvae were then measured. The mean values for haemolymph osmotic pressure and conductivity are given in Fig. 1 (mean values for sodium are given in Fig. 2). In these larvae the haemolymph was slightly but significantly hypo-osmotic to external concentrations of 275, 330 and 410 mM./l. NaCl. Respective values for t are 6·08, 4·89 and 4·34; in all cases $P < 0{\cdot}001$. In similar experiments, however, the haemolymph was not always significantly hypo-osmotic to the medium, and in some individuals it was distinctly hyper-osmotic. Altogether, measurements of haemolymph osmotic pressure in ninety larvae kept at external concentrations varying between 220 and 410 mM./l. NaCl showed that 32% were either iso- or hyper-osmotic to the medium. At an external concentration of 472 mM./l. NaCl the haemolymph in the majority of larvae was slightly hyper-osmotic.

It was possible that the artificial conditions of these experiments, such as starvation, confinement in a small volume of medium and the absence of the protective case, may have upset the normal regulatory mechanism and caused the haemolymph to become

slightly hypo-osmotic to the medium. This was examined by keeping larvae under as natural conditions as possible in tanks containing 275, 330 and 410 mM,/l. NaCl respectively. The results of osmotic pressure measurements on haemolymph samples from these larvae were very similar to those discussed above, and the range of variation between hypo- and hyper-osmoticity was just as great. It was concluded, therefore, that the experimental conditions were not affecting the results, and that the haemolymph of *L. affinis* larvae may be either slightly hypo- or hyper-osmotic at these external concentrations. This point will be referred to again in the discussion.

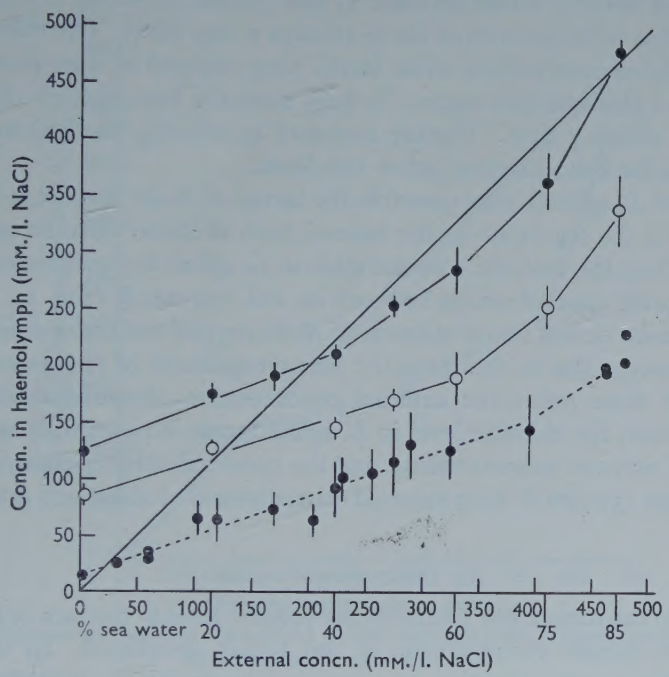

Fig. 1. The relation between the external concentration and the concentration in haemolymph; ●—●, osmotic pressure, each point represents the mean value of haemolymph samples from six or seven larvae; ○—○, conductivity of the above haemolymph samples; ●– – –●, chloride concentration, mean values of haemolymph samples from five to twenty-four larvae, except at an external concentration of 60 mM./l. NaCl and concentrations above 450 mM./l. NaCl, where values are for individual larvae.

The rise in haemolymph osmotic pressure of larvae kept in sea-water media is not accounted for simply by an increase in the total ionic fraction of the haemolymph, estimated by measurements of the conductivity (Fig. 1). Thus it appears that the non-electrolyte fraction of the haemolymph is increased when the external salt concentration is raised. At an external concentration of 410 mM./l. NaCl the non-electrolyte fraction in the haemolymph was approximately three times greater than for larvae kept in tap water. This increase is not due to changes in water content of the larvae. The total water content, estimated by difference between wet and dry weights of whole larvae, remained constant at 80–85 % of the wet weight at external concentrations ranging from tap water to 410 mM./l. NaCl. This suggests that the increase in concentration of the non-electrolyte fraction is brought about by the liberation of osmotically

active substances into the haemolymph. Such a mechanism might, for example, involve the mobilization of amino acids from a protein reservoir, as occurs in the muscle fibres of *Carcinus maenas* (Shaw, 1958) and *Potamon niloticus* (Shaw, 1959).

IV. IONIC REGULATION

(*a*) *Haemolymph chloride*

The chloride concentration in the haemolymph of larvae kept in tap water is very low. In well fed larvae it varied between 15 and 30 mM./l., falling to a mean value of 12 mM./l. chloride in larvae starved for 6–10 days in tap water. The chloride concentration was reduced even further when larvae were exposed to slow-running streams of tap water and glass-distilled water. In both cases the haemolymph chloride fell to about 5 mM./l. within 7 days. Further exposure to running distilled water failed to reduce the chloride concentration below this level.

In this respect *L. affinis* larvae resemble the larvae of *Sialis* (Beadle & Shaw, 1950), but they differ in the regulation of the haemolymph chloride when larvae are kept in saline media. Then the chloride concentration in *L. affinis* larvae remained hypotonic at external chloride concentrations between 40 and 100 mM./l. (Fig. 1). This kind of regulation is similar to that found in larvae of *Aëdes aegypti* and *Culex pipiens* (Wigglesworth, 1938), except that in the mosquito larvae regulation of haemolymph chloride began to break down when the external concentration exceeded about 120 mM./l. NaCl. In contrast, the chloride level in *L. affinis* larvae is powerfully regulated over a wide range of external concentrations, and the mean value for haemolymph chloride was still less than 150 mM./l. at an external concentration of about 400 mM./l. chloride.

(*b*) *Haemolymph sodium*

The ability to maintain a low haemolymph sodium level in the face of large changes in the external sodium concentration is also highly developed. In tap water the haemolymph sodium concentration, at a little below 100 mM./l., is similar to the level found in larvae of *Sialis* (Shaw, 1955*b*) and *A. aegypti* (Ramsay, 1953). However, *L. affinis* differs from these fresh-water insects in that the haemolymph sodium became strongly hypotonic at external sodium concentrations greater than about 150 mM./l. (Fig. 2).

The degree of regulation is such that at an external concentration of 370 mM./l. the haemolymph sodium level is only slightly more than doubled. At an external sodium concentration of 423 mM./l., however, there was a marked increase in the haemolymph sodium level. At this concentration (approximately 85 % sea water) the majority of larvae did not survive for more than a few days. The mean value of 356 mM./l. for haemolymph sodium (Fig. 2) was obtained from larvae kept at 423 mM./l. sodium for only 3 days, and it is possible that the haemolymph sodium would have increased further to become isotonic before the final death of the larvae.

Since the ionic fraction of the haemolymph is considerably hypotonic at external concentrations up to at least 410 mM./l. NaCl, it is important to establish whether or not this is due to an impermeable body wall. The following experiments were carried out to investigate this point.

(*c*) *Permeability to chloride*

Two groups of larvae were first adapted to tap water in order to lower the haemolymph chloride concentration. After 5 days in tap water group A was then transferred to an external chloride concentration of 116 mM./l. and group B to 222 mM./l. Before transference to these media some of the larvae were ligatured as indicated in Table 1. Changes in body weight were followed during the course of the experiment, and the haemolymph chloride concentration was determined after 7 days (group A) and 9 days (group B) in their respective media (Table 1).

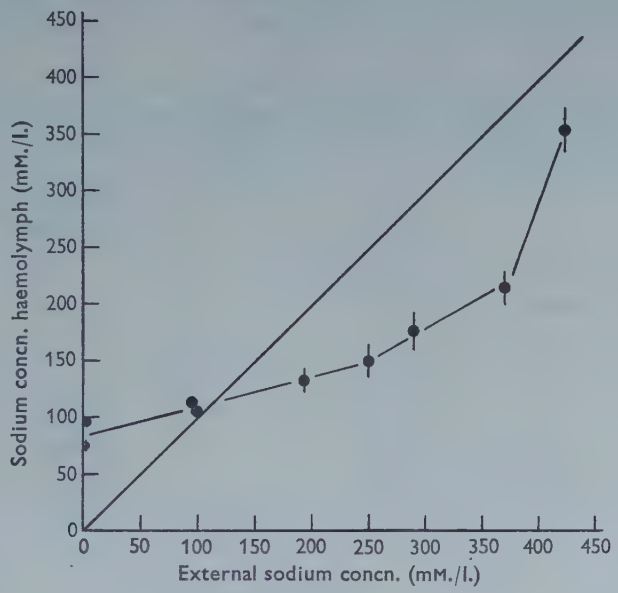

Fig. 2. The relation between the sodium concentration in haemolymph and the sodium concentration in sea-water media.

In both groups there was fairly good agreement between the values for haemolymph chloride in larvae prevented from drinking the medium. In these larvae, with the exception of serials 149 and 166, the chloride concentration was approximately one half that of the controls, and was well below the normal range of haemolymph concentrations found in other larvae kept at 116 and 222 mM./l. chloride (Fig. 1). Now in tap water the mean value for haemolymph chloride concentration is 12 mM./l. (§ IV(*a*)). After 7 days at 116 mM./l. and 9 days at 222 mM./l. chloride the mean haemolymph chloride concentration in larvae prevented from drinking the medium had risen to 24 and 43·5 mM./l. respectively (excluding serial 166). In group A the concentration difference between haemolymph and medium at the start of the experiment was 116 − 12 = 104 mM./l. chloride, and in 7 days the haemolymph chloride had increased by 24 − 12 = 12 mM./l. This is equivalent to an increase in haemolymph chloride concentration by 1·7 mM./l. per day. Similarly, in group B, at an initial concentration difference of 210 mM./l. chloride, the haemolymph chloride concentration was increased by 3·5 mM./l. per day.

These results indicate that the body wall of *L. affinis* larvae is relatively impermeable

to chloride. In more critical experiments Shaw (1955*a*) demonstrated that the cuticle of *Sialis* larvae is also relatively impermeable to chloride. From the data given in fig. 6 of Shaw's paper we can calculate that, at a concentration difference of about 50 mM./l. chloride, the inward diffusion of chloride during the first 7 days raised the haemolymph concentration in *Sialis* by approximately 3 mM./l. per day at 17° C. Similarly, at a concentration difference of about 140 mM./l. the haemolymph chloride was raised by approximately 6 mM./l. per day at 17° C. Thus the body wall of *L. affinis* larvae appears to be even less permeable to chloride than the body wall of *Sialis* larvae. In view of the greater permeability to water of the body wall in *L. affinis* compared with *Sialis* (see below, § V(*a*)) it would be of some interest to carry out more critical estimations of permeability to chloride in caddis larvae.

Table 1. *Percentage changes in weight, and the haemolymph chloride concentration in larvae after 7 days in* 116 mM./l. *chloride (group A), and after 9 days in* 222 mM./l. *chloride (group B)*

Serial	Treatment	Percentage change in body weight	Haemolymph chloride (mM./l.)
	Group A		
137	None	0	43
138		−1	39
139	Mouth sealed*	−5	24
140		−4	21
141		−3	27
142		−16	19
144	Abdominal ligature†	+10	34
146		+19	19
147	Mouth sealed and abdominal ligature	+15	24
148		+9	15
149		+9	37
	Group B		
163	None	−4	77
164	Mouth sealed*	−15	43
165		−14	34
166		−6	75
167	Abdominal ligature†	+3	82
168		+21	108
169		+2	84
170	Mouth sealed and abdominal ligature	−4	46
171		−4	51

* Sealed with wax to prevent larva from drinking the medium.
† Ligatured with fine thread between penultimate and terminal abdominal segments.

It will be shown later that when larvae adapted to tap water are transferred to sea-water media the haemolymph chloride concentration reaches its equilibrium level with the external concentration in about 2–3 days. At an external concentration of 120 mM./l. the equilibrium level for haemolymph chloride is 62 ± 19 mM./l. and at 222 mM./l. the equilibrium level for haemolymph chloride is 89 ± 26 mM./l. (Fig. 1). These increases must be due largely to uptake of chloride through the gut wall, since diffusion through the body wall in 2 days would account for only about 10% of the total increase in haemolymph chloride during that period.

(*d*) *Permeability to sodium*

Permeability of the body wall to sodium was investigated by measuring the influx of ^{24}Na from sea-water media, using larvae with the mouth sealed to prevent drinking. Following the generally accepted practice, a larva is considered here to be a single-compartment system (the haemolymph) containing all of the exchangeable sodium in the animal (see Treherne, 1954*b*). After the addition of a relatively minute quantity of labelled sodium to the external medium, the exchange of labelled sodium between medium and haemolymph should increase asymptotically until equilibrium is reached. The rate of exchange will be proportional to the ratio C_i/C_o where C_i is the internal concentration and C_o is the external concentration of unlabelled sodium. Thus

$$A_i = A\frac{C_i}{C_o}(1-e^{-t/T}),$$

where A is the radioactivity of the medium, T the 'time constant' for exchange of labelled sodium, and A_i is the radioactivity of the haemolymph at the time t.

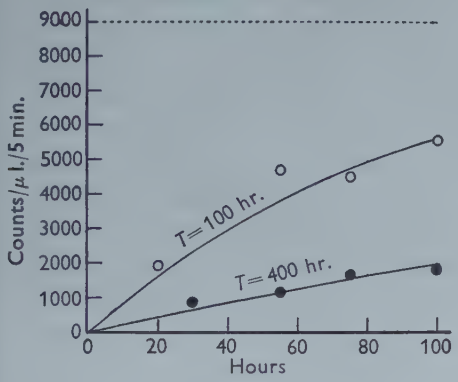

Fig. 3

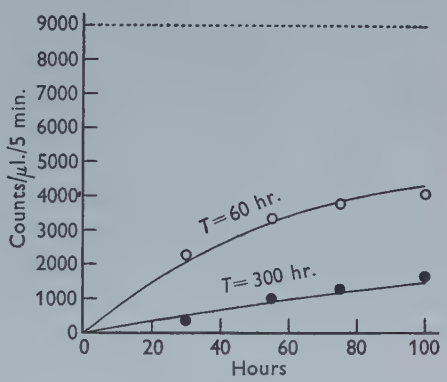

Fig. 4

Fig. 3. Influx of ^{24}Na into the haemolymph of normal larvae, ○, and larvae with the mouths sealed, ●, in a sea-water medium containing 100 mM./l. sodium. The horizontal broken line indicates the concentration of ^{24}Na in the sea-water medium.

Fig. 4. Influx of ^{24}Na into the haemolymph of normal larvae, ○, and larvae with the mouths sealed, ●, in a sea-water medium containing 300 mM./l. sodium. The horizontal broken line indicates the concentration of ^{24}Na in the sea-water medium.

At intervals, haemolymph radioactivity was measured in two to four larvae prevented from drinking the medium and in a similar number of normal larvae. The results from larvae at an external sodium concentration of 100 mM./l. are shown in Fig. 3, together with two theoretical curves describing exponential increases in haemolymph radioactivity when $T = 400$ and $T = 100$ hr. respectively, and $C_i =$ 100 mM./l. sodium (from Fig. 2).

In larvae prevented from drinking the medium the measured influx of ^{24}Na is approximately described by the curve where $T = 400$ hr. or about 17 days. Since $C_i =$ 100 mM./l. sodium, the rate of sodium influx through the body wall is roughly equivalent to that required to raise the haemolymph sodium concentration by 6 mM./l. per day if C_i was zero. This may be compared with the rate of chloride influx through

the body wall, at a similar concentration difference, equivalent to approximately 2 mM./l. chloride per day. The influx rates of the two ions are at least of the same order, and it would be unwise to claim that the higher rate of sodium influx is significant until more critical measurements of chloride influx are available. However, it is worth stating at this stage that unpublished measurements suggest that a small proportion of the ^{24}Na influx at an external concentration of 100 mM./l. sodium represents active transport of sodium.

Fig. 4 shows ^{24}Na influx through the body wall of larvae at an external sodium concentration of 300 mM./l. The experimental results best fit an exponential curve where $T = 300$ hr. or 12·5 days, and $C_i = 180$ mM./l. sodium. Hence the influx rate is equivalent to that required to raise the haemolymph sodium concentration by about 14·5 mM./l. per day if C_i was zero. This influx is two to three times greater than the rate of 6 mM./l. per day at an external sodium concentration of 100 mM./l. Now the rate of inward diffusion of unlabelled sodium will be directly proportional to the external sodium concentration. If ^{24}Na influx is also a measure of the rate of inward diffusion of unlabelled sodium, then the influx rate of ^{24}Na from the above two experimental media should differ by a factor of three. Bearing in mind the possibility of some active transport from 100 mM./l. sodium, the two values for ^{24}Na influx are in very good agreement.

Since the influx rate of sodium is similar to that for chloride, it is apparent that the body wall of *L. affinis* larvae is relatively impermeable to both chloride and sodium ions. Furthermore, as was indicated above for chloride (§ IV(*c*)), the major uptake of sodium from sea-water media occurs through the gut wall. ^{24}Na influx from 100 and 300 mM./l. sodium in normal larvae is shown in Figs. 3 and 4 respectively, and it is immediately clear that the rate of influx is far greater than in larvae prevented from drinking the medium. Further discussion of these results is deferred to a later paper.

(*e*) *Chloride concentration of the rectal fluid*

Excretion of chloride was investigated in the following way. Nine larvae (serials A–I) were removed from tanks containing approximately 30% sea water and were placed individually in tubes containing tap water, changed every 48 hr. After 57 hr. in tap water the chloride concentration in the rectal fluid of six larvae was determined, and was measured again at intervals until no chloride could be detected (Table 2). After 8 days in tap water all nine larvae were transferred directly into sea water containing 256 mM./l. chloride and the chloride output in the rectal fluid was measured (Table 3). At various intervals larvae were removed from the experiment and the haemolymph chloride concentration was also determined. Serial I died during the second part of the experiment and is not included in Table 3.

Three main conclusions may be drawn from Table 3.

(1) When larvae were transferred from tap water into 256 mM./l. chloride the concentration in the rectal fluid increased rapidly from 0 to about 200 mM./l. chloride within 48 hr.

(2) Larvae elaborate rectal fluid in which the chloride concentration is at least treble the concentration in the haemolymph.

(3) The rectal fluid can be hypertonic to the external medium.

It will also be seen from Table 3 that the haemolymph chloride had increased to the final equilibrium level in 2 days. In similar experiments at other external concentrations the haemolymph chloride also reached equilibrium within 2–3 days. Since diffusion through the body wall would account for only some 10–15 % of the observed increase in haemolymph chloride, it is clear that changes in haemolymph chloride level when larvae are placed in sea-water media are due mainly to drinking and absorbing salts through the gut wall.

Table 2. *The chloride concentration in rectal fluid of larvae kept in tap water (see text)*

Hours in tap water	Chloride concentration (mM./l.) in rectal fluid					
57	3	50	46	50	86	50
80	0	3	7	29	60	12
144	–	0	–	10	0	2
192	0	0	0	0	0	0
Serial	C	D	E	F	G	I

Table 3. *The chloride concentration in rectal fluid and haemolymph of larvae transferred from tap water into* 256 mM./l. *chloride*

Hours in 256 mM./l. Cl	Chloride concentration (mM./l.) in rectal fluid and in haemolymph*							
24	—	—	—	26	—	114	45	60
48	228 (110)	198 (57)	— (86)	—	—	—	178	—
72	—	—	—	137 (60)	202 (110)	204	192	—
120	—	—	—	—	—	—	272 (103)	—
168	—	—	—	—	—	268 (69)	—	256 (94)
Serial	A	B	C	D	E	F	G	H

* Haemolymph chloride concentrations are given in parentheses.

The main points arising out of this section can now be briefly summarized as follows. The salt concentration in the haemolymph is maintained at a low level over a wide range of external concentrations. This is due partly to the low salt permeability of the body wall. Most of the salt uptake occurs via the mouth, and excess salts are eliminated in the excretory fluid. The slight but gradual increase in the haemolymph-salt equilibrium level at increasingly higher external salt concentrations probably reflects new balance conditions established between salt uptake and output. Most of the increased salt uptake (in terms of concentration, mM./l. NaCl) is balanced by elaborating rectal fluid in which the salt concentration is slightly greater than that of the external medium.

V. WATER BALANCE

At external concentrations above 200 mM./l. NaCl the salt concentration in the haemolymph is maintained at a level considerably lower than the concentration in the medium. On the other hand, the total concentration of the haemolymph increases

to a level approximately equivalent to that of the medium. This behaviour is very different from that of the salt-water mosquito larva *Aëdes detritus*, in which the haemolymph remains strongly hypo-osmotic to external concentrations above 200 mM./l. NaCl (Beadle, 1939). Beadle demonstrated that the body wall of *A. detritus* larvae is practically impermeable, not only to salts but also to water. In the majority of *L. affinis* larvae the haemolymph is apparently slightly hypo-osmotic to high external concentrations. It is therefore important to establish whether the body wall of *L. affinis* larvae is also impermeable to water.

(*a*) *Permeability to water*

This was estimated by measuring changes in wet weight of larvae adapted to tap water. In one experiment of this kind two larvae were prevented from drinking tap water by sealing the mouths with wax, and were also ligatured with fine thread between

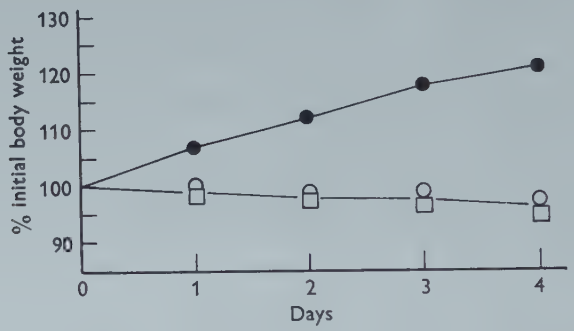

Fig. 5. The percentage change in body weight of larvae in tap water. ●, larvae with mouths sealed and abdominal ligatures; ○, normal larvae; □, larvae with mouths sealed. Each point represents the mean weight of two larvae.

the penultimate and terminal abdominal segments (abdominal ligature). The daily increase in weight of these larvae in tap water was compared with that in two normal larvae and two larvae with the mouths sealed but no abdominal ligature. The results are shown in Fig. 5.

During the first 24 hr. the increase in weight by osmotic uptake of water through the body wall was equivalent to 7% of the wet weight. This crude estimate indicates that the cuticle of *L. affinis* is roughly twice as permeable to water as the cuticle of *Sialis* larvae, where the osmotic uptake was approximately 4% of the body weight per day at 20° C. (Shaw, 1955*a*).

Holdgate (1956), measuring rates of water loss, also found that the cuticle of *Limnephilus* larvae from fresh water was nearly twice as permeable to water as in *Sialis* larvae. This was confirmed by repeating the experiment described above for *L. affinis* on two species of caddis larvae obtained from freshwater habitats, viz. *Limnephilus stigma* and *Anabolia nervosa*. In both species the osmotic uptake of water in 24 hr. was approximately equivalent to 7% of the wet weight.

It is clear that the body wall in *L. affinis* larvae is not impermeable to water. In fact, its permeability is very similar to that in freshwater species of caddis larvae. Furthermore, it is greater than the permeability of a wide variety of other freshwater insects

(see Holdgate, 1956). It follows that, if the haemolymph is maintained slightly hypo-osmotic to the medium, there will be a small but steady osmotic loss of water. This osmotic loss must be offset by gaining a similar amount of osmotically free water from the medium.

(*b*) *Osmotic pressure of the rectal fluid*

The rectal fluid of larvae kept in tap water was considerably hypo-osmotic to the haemolymph, but when the haemolymph osmotic pressure was raised by placing larvae in sea-water media, the osmotic pressure of the rectal fluid increased rapidly (Fig. 6). At a haemolymph osmotic pressure of about 200 mM./l. NaCl the rectal

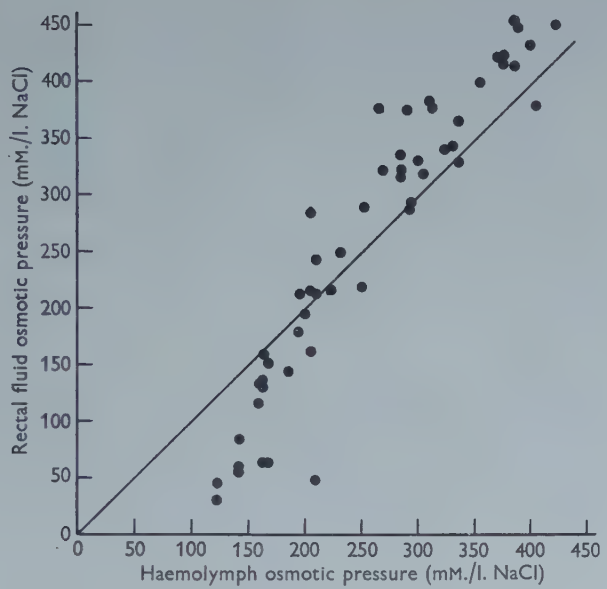

Fig. 6. The relation between the osmotic pressures of rectal fluid and haemolymph.

Table 4. *Analysis for significant difference by 't' test*

A. Difference between the mean osmotic pressures (mM./l. NaCl) of rectal fluid and haemolymph

N	Haemolymph mean O.P.	S.D.	Rectal fluid mean O.P.	S.D.	Difference	Significance levels	Medium O.P. (mM./l. NaCl)
15	306	25	347	31	41	$t = 3{\cdot}99$ $P < 0{\cdot}001$	330
6	393	19	439	17	54	$t = 5{\cdot}19$ $P < 0{\cdot}001$	400–408

B. Difference between the mean osmotic pressures (mM./l. NaCl) of rectal fluid and medium

N	Medium mean O.P.	S.D.	Rectal fluid mean O.P.	S.D.	Difference	Significance levels
15	330	0	347	31	17	$t = 2{\cdot}12$ $P = 0{\cdot}05$–$0{\cdot}02$
6	404	3	439	17	35	$t = 4{\cdot}99$ $P < 0{\cdot}001$

fluid became iso-osmotic, and at higher haemolymph concentrations the rectal fluid was concentrated to become significantly hyper-osmotic to the haemolymph (Table 4A). Furthermore, the rectal fluid was slightly but significantly hyper-osmotic to the medium at external concentrations of 330 and 404 mM./l. NaCl (Table 4B).

Now it was shown above (§ IV(*e*)) that at high external salt concentrations the rectal fluid chloride can be hypertonic to the medium. It was also shown that larvae drink sea-water media. Since the excretory fluid is hyper-osmotic to the medium which is swallowed, it follows that a small quantity of osmotically free water (Smith, 1956, p. 109) can be gained by drinking salt water and excreting it as a concentrated solution of salts. This osmotically free water will then be available to offset a small osmotic loss through the body wall.

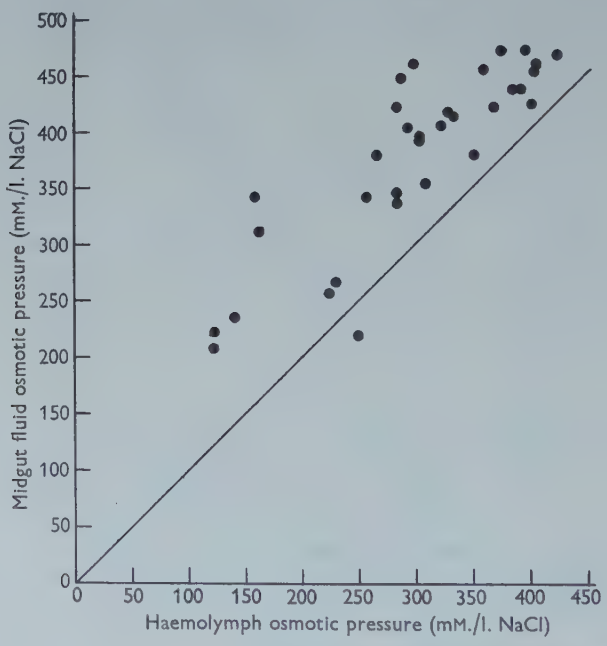

Fig. 7. The relation between the osmotic pressures of midgut fluid and haemolymph.

(*c*) *Osmotic pressure of the midgut fluid*

Midgut fluid was obtained from larvae adapted to tap water and to various external concentrations of sea water. Larvae were narcotized with CO_2, washed rapidly in distilled water, and dried with filter paper. Each larva was then sealed firmly into a wax block by melting the wax immediately surrounding the larva. A median dorsal incision exposed the alimentary canal, and at the same time a sample of haemolymph was taken for analysis. With a pair of fine forceps the midgut was then lifted clear of the haemolymph, and excess haemolymph carefully removed with filter-paper. A fine glass pipette containing a column of liquid paraffin was thrust through the midgut wall and the fluid contents (usually 1–3 μl.) were sucked into the pipette behind the column of paraffin. The tip of the pipette was sealed in a micro flame, and the fluid was then centrifuged to throw down particulate matter. In most larvae the

midgut fluid contained a dark brown pigment in solution, but in a few instances this pigment was entirely absent. The supernatant was used to compare the osmotic pressure of the midgut fluid with that of the haemolymph. The results are given in Fig. 7.

With one exception the midgut fluid was hyper-osmotic to the haemolymph. The difference in osmotic pressure of the two body fluids was particularly striking when larvae were kept in tap water; the mean value for midgut fluid was 223 mM./l. NaCl, whereas that for the haemolymph was only 130 mM./l. NaCl. The midgut fluid was also significantly hyper-osmotic to media varying in concentration from 0 to 405 mM./l. NaCl (Table 5).

Table 5. *Analysis by 't' test for significant differences between the mean osmotic pressures* (mM./l. NaCl) *of midgut fluid and medium*

N	Medium mean O.P.	S.D.	Midgut fluid mean O.P.	S.D.	Difference	Significance levels
3	Tap water	—	223	—	223	—
3	233	—	303	—	70	—
13	330	0	388	48	58	$t = 4{\cdot}35$ $P < 0{\cdot}001$
10	405	3	436	31	31	$t = 3{\cdot}29$ $P = 0{\cdot}01$–$0{\cdot}001$

The significance of maintaining a hyper-osmotic midgut fluid is obscure. Similar results were obtained with the freshwater caddis larvae *Limnephilus stigma* and *Anabolia nervosa* (Sutcliffe, 1961*b*). This suggests that, although the osmotic differences between the two body fluids may be exploited in maintaining salt and water balance, the differences are more fundamentally concerned with other processes in the midgut, e.g. digestion and absorption.

DISCUSSION

Since the body wall is highly permeable to water it would clearly be difficult, if not impossible, to maintain the haemolymph markedly hypo-osmotic to the medium. On the other hand, the salt-regulatory mechanism is apparently concerned with maintaining a low salt concentration in the haemolymph. These conflicting requirements of salt and water balance appear to have been met by a compromise, i.e. the ability to increase the non-electrolyte fraction in the haemolymph. It is interesting to note that a very similar situation exists in marine elasmobranch fishes. Here, although the blood salt level is strongly hypotonic to sea water, osmotic loss of water is prevented by the retention of urea in high concentrations, so that the blood is maintained slightly hyper-osmotic to sea water (Smith, 1936).

It was suggested (§ III) that the non-electrolyte fraction in the haemolymph is increased by mobilizing osmotically active substances from a protein reservoir. It is quite possible that a mechanism of this kind is not so highly developed in some larvae as in others, and this may partly explain individual differences in maintaining the haemolymph slightly hyper- or hypo-osmotic to the medium at high external salt concentrations. However, it is also possible that the regulation of salt and water

content is so delicately balanced that, in fact, there are small, regular oscillations of the haemolymph osmotic pressure within certain limits above and below the osmotic pressure of the medium. These oscillations could, for example, reflect a regular intake of salt water by drinking, followed by its removal from the haemolymph and elimination as a concentrated solution of salts. Until more is known about the regulatory mechanism in *L. affinis* larvae it seems best to regard the haemolymph as roughly iso-osmotic at high external salt concentrations.

Ramsay (1954) has already suggested that the inability of many freshwater animals to survive in salt concentrations greater than the normal blood level may be due to an inability to increase the amino acid content of the muscle cells. In the case of *L. affinis* it seems highly probable that, as in the haemolymph, the osmotic level of tissue cell fluid is also raised by increasing the non-electrolyte fraction. It may be suggested here that the maintenance of a low haemolymph salt concentration is concerned with the regulation of a low salt concentration in the tissue cells, whereas the high haemolymph osmotic pressure is necessary to prevent general dehydration. In any case, it is clear that survival of *L. affinis* larvae in high salt concentrations must have involved adaptation of the tissue cells to withstand osmotic pressures at least three times greater than those existing in freshwater larvae. Such adaptation may be a common feature of salt-water insects. *Aëdes detritus* larvae survive, and even pupate when the haemolymph osmotic pressure is roughly trebled following penetration of the non-electrolyte glycerol (Beadle, 1939). Beadle stressed that the tissues must be extremely resistant to changes in concentration and composition of the haemolymph, and that this resistance must be regarded as part of the adaptive mechanism for survival in salt water.

The maintenance of a low haemolymph salt level against large external concentration gradients is due to the low salt permeability of the body wall, and also to the activity of the Malpighian tubule-rectal system. This system elaborates an excretory fluid in which the chloride concentration is more than treble the concentration in the haemolymph. Moreover, the system is very sensitive to changes in the haemolymph chloride level; an increase in this level is rapidly followed by a rise in the rectal fluid concentration. This feature is of some importance to larvae living in salt-marsh pools, where an increase in salinity is usually brought about by sudden influxes of sea water. These influxes sometimes occur after long periods of relatively low-salinity conditions (Sutcliffe, 1961*a*) and larvae are therefore exposed to large and very rapid increases in salt concentration.

The ability to produce an excretory fluid both hypertonic and hyperosmotic, not only to the haemolymph but also to the external medium, is a feature of major importance to an animal living in high salt concentrations. By the relatively simple process of drinking salt water and concentrating the salts in the excretory fluid, a small quantity of osmotically free water can be obtained from the medium. One advantage of this process is that a regular flow of water (containing salts) can be maintained as a vehicle for the removal of metabolic waste products. A second and probably more important advantage is that the osmotically free water obtained from the medium can be used to combat the continuous threat of dehydration. Unless osmotic loss of water is offset in some way, dehydration of the haemolymph, and hence of the tissue cells, is inevitable in an animal with a cuticle permeable to water. In larvae of *Sialis* (Shaw,

1955*b*) and *Aëdes aegypti* (Wigglesworth, 1938), both of which are permeable to water, the regulation of salt and water balance begins to break down when the external medium is made iso-osmotic with the haemolymph. Neither of these insects can produce a concentrated fluid in the rectum. In striking contrast, although the body wall of *L. affinis* larvae is highly permeable to water, salt and water regulation does not break down when the external medium is made iso-osmotic with the haemolymph. Indeed, larvae tolerate external (and internal) concentrations more than treble the haemolymph osmotic pressure of tap-water larvae.

At this point is it convenient to mention the work of Claus (1937) on regulation in adult *Corixa* (= *Sigara*). Claus investigated *C. lugubris*, a brackish-water corixid occurring in salinities up to at least 18‰, and the freshwater species *C. distincta* and *C. fossarum*. He found that the haemolymph osmotic pressure in the freshwater species increased gradually over a range of external salinities from 1‰ to about 19‰, in which the haemolymph became roughly iso-osmotic. On the other hand, in *C. lugubris* the haemolymph osmotic pressure remained constant over the external range 1–18‰ salinity, then increased gradually in salinities up to about 22‰. At external salinities above 14‰ the haemolymph in *C. lugubris* was hypo-osmotic. These findings must be viewed with some caution. As Krogh (1939) has already pointed out, it is doubtful whether the animals were given sufficient time to reach equilibrium with new external concentrations. Acclimatization periods of sometimes considerably less than three days duration seem far too short for insects in which the permeability to water is several times lower than in *Limnephilus* and *Sialis* larvae (Holdgate, 1956).

Finally, the main difference apparent between *L. affinis* and other salt-water insects is the ability of the latter to maintain the haemolymph strongly hypo-osmotic to high external salt concentrations. In *Aëdes detritus* larvae this must be due largely to a cuticle impermeable both to water and salts, since the ability to elaborate rectal fluid more concentrated than the medium appears to be no better developed than in *L. affinis* larvae (cf. Ramsay, 1950). Larvae of *Ephydra riparia*, however, elaborate rectal fluid considerably more concentrated than the external medium (Sutcliffe, 1960*a*). Like *E. riparia*, larvae of *E. cinerea* also remain strongly hypo-osmotic to very high external salt concentrations, in spite of the fact that the cuticle is very slightly permeable to water, and possibly also to salts (Nemenz, 1960). In this case the production of a highly concentrated excretory fluid must be a necessary part of the hypo-osmotic regulatory mechanism in *Ephydra* larvae.

SUMMARY

1. *Limnephilus affinis* larvae tolerate external salt concentrations up to at least 410 mM./l. NaCl (about 75 % sea water) and survive for short periods in 470 mM./l. NaCl (about 85 % sea water).

2. The body wall is highly permeable to water, but relatively impermeable to sodium and chloride. Most of the sodium and chloride uptake from salt water occurs via the mouth.

3. The sodium and chloride levels in the haemolymph are powerfully regulated. Both are maintained strongly hypotonic against large external concentration gradients.

4. The Malpighian tubule-rectal system is very sensitive to changes in the haemolymph chloride level. The chloride concentration in the rectal fluid can be at least three times greater than the concentration in the haemolymph, and slightly greater than the concentration in the external medium.

5. The rectal fluid is hyper-osmotic to the haemolymph and to the medium at high external salt concentrations.

6. At external concentrations greater than about 200 mM./l. NaCl, water balance is maintained by regulating the haemolymph roughly iso-osmotic with the medium. This is partly achieved by increasing the non-electrolyte fraction in the haemolymph. A small quantity of osmotically free water is available to replace any osmotic loss. This can be obtained by drinking salt water and producing a concentrated solution of salts in the rectum.

It is a pleasure to thank Mr J. Shaw for guiding me into this field of research, and for his generous advice and criticisms at all stages of the work. I am indebted to the Department of Scientific and Industrial Research for a maintenance grant.

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J. Exp. Biol. (1961), **38**, 521–530
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STUDIES ON SALT AND WATER BALANCE IN CADDIS LARVAE (TRICHOPTERA)

II. OSMOTIC AND IONIC REGULATION OF BODY FLUIDS IN *LIMNEPHILUS STIGMA* CURTIS AND *ANABOLIA NERVOSA* LEACH

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INTRODUCTION

Only two species of caddis larvae are known to occur in water with a salt concentration approaching that of normal sea water. Larvae of *Philanisus plebeius* Walker live in rock pools on the coasts of New Zealand and Australia (McLachlan, 1882; Hudson, 1904), and larvae of *Limnephilus affinis* Curtis live in salt marsh pools on the coast of Britain (Sutcliffe, 1960). All other records of caddis larvae in salt water concern brackish waters with a salt concentration less than that of 30% sea water. Osmoregulation of the body fluids in *L. affinis* larvae was described in the first paper of this series (Sutcliffe, 1961). The object of the work reported here was to investigate osmoregulation of the body fluids in caddis larvae which normally occur only in fresh water, in order to compare their regulatory mechanism with that of the euryhaline larvae of *L. affinis*.

Two species of freshwater caddis larvae were used; *L. stigma* Curtis, which occurs in ponds and lakes, and *Anabolia nervosa* Leach, common in slow-flowing reaches of rivers. Regulation of the haemolymph chloride level has already been studied in one other freshwater caddis larva, *L. flavicornis* Fabricius (Boné & Koch, 1942). However, these authors only studied the chloride regulation of larvae kept in salt solutions in which the chloride concentrations were roughly equivalent to the range of concentrations found in natural fresh waters.

The osmoregulatory mechanisms of two other freshwater insects kept in saline media have already been studied in some detail. Wigglesworth (1933, 1938) and Ramsay (1950, 1951, 1953) worked with larvae of *Aëdes aegypti*. Beadle & Shaw (1950) and Shaw (1955) investigated larvae of *Sialis lutaria*. Chloride regulation has also been investigated in larvae of *Helodes* (Treherne, 1954) and in larvae of *Aeschna* and *Libellula* (Schoffeniels, 1950).

MATERIAL AND METHODS

Limnephilus stigma larvae were obtained from a small pond in Gosforth Park, Northumberland. *Anabolia nervosa* larvae were obtained from the River Blyth. Imagines of both species were reared out in the laboratory to confirm the identifications of the larvae.

Body fluids were removed and analysed as described previously (Sutcliffe, 1961). Larvae were not fed during the course of experiments, and usually were not removed from their cases until body fluids were required for analysis. Larvae were kept individually in beakers containing about 100 ml. of the experimental medium. Most of the experiments were carried out at 14–17° C.

Media were Newcastle tap water and local sea water from Cullercoats diluted with tap water to the required concentration. As in the previous paper, sea-water media are referred to in terms of equivalent concentrations of sodium chloride (mM./l. NaCl).

SURVIVAL IN SEA-WATER MEDIA

The majority of larvae of both species did not survive for more than a few days at external salt concentrations greater than about 60 mM./l. NaCl. Mortality was particularly high when larvae were transferred directly from tap water into high salt concentrations. Thus in 120 mM./l. NaCl about 50% of the larvae died within 3 days. In 170 mM./l. NaCl about 75% died within 3 days and in 220 mM./l. NaCl only a few individuals survived for more than 2 days.

In order to obtain measurements on the body fluids of larvae kept in 170–220 mM./l. NaCl it was necessary to increase the salt concentration gradually over a period of about 7 days. Survival was also slightly increased by lowering the temperature to 10–12° C. Even so, more than 50% of the larvae died during the process, and none survived in 220 mM./l. NaCl for more than 6–7 days. Body fluids of larvae kept in concentrations above 120 mM./l. NaCl were usually analysed after 2–3 days at the final experimental salt concentration. In considering the following results it should be remembered that they were obtained only from those few larvae which were relatively successful in surviving the higher salt concentrations.

RESULTS

(*a*) *Haemolymph osmotic pressure and conductivity*

The relationship between osmotic pressure of the haemolymph and that of the medium is shown in Fig. 1A (*L. stigma*) and Fig. 1B (*A. nervosa*). The normal haemolymph osmotic pressure in both species is relatively low. The mean value for six *L. stigma* larvae was 102 ± 5 mM./l. NaCl, and the mean value for six *A. nervosa* larvae was 113 ± 10 mM./l. NaCl.

In sea-water media the haemolymph osmotic pressure increased gradually and remained slightly hyper-osmotic to the medium. This increase was due entirely to an increase in the electrolyte fraction of the haemolymph, estimated by measurements of its conductivity (Table 1). In this respect both species differ from *L. affinis* larvae, in which the electrolyte fraction of the haemolymph did not increase to the same extent as the haemolymph osmotic pressure (Sutcliffe, 1961).

(*b*) *Haemolymph chloride*

In both species there was considerable individual variation in the extent to which the haemolymph chloride concentration was regulated in sea-water media (Fig. 1A, B). In some larvae the chloride concentration increased to contribute practically all of the anion fraction of the haemolymph. Nevertheless, in the majority of larvae a low

chloride level was maintained against fairly high external concentration gradients, and in all cases the haemolymph remained hypotonic with respect to chloride until just prior to death.

Table 1. *Mean values of the haemolymph osmotic pressure and conductivity in larvae of* Limnephilus stigma *and* Anabolia nervosa *at different external salt concentrations*

		mM./l. NaCl				
		Haemolymph		Conductivity		Medium
Species	*N*	O.P.	S.D.		S.D.	O.P.
L. stigma	4	101	—	88	—	Tap water
	6	154	14	148	7	108
	4	199	—	204	—	190
	2	232	—	228	—	226
A. nervosa	2	125	—	108	—	Tap water
	6	189	9	176	8	178
	5	233	26	193	14·5	220

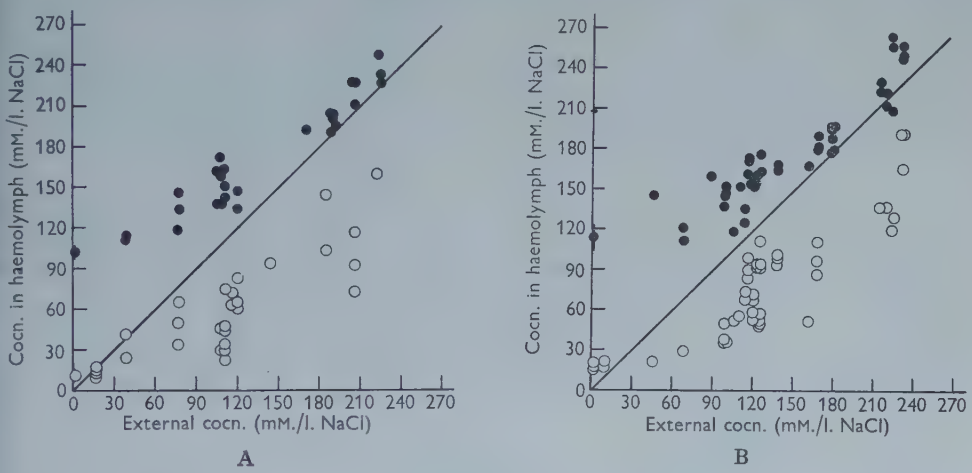


Fig. 1. The relation between the external concentration and the concentration in haemolymph of A, *L. stigma* and B, *A. nervosa* larvae. ●, Haemolymph osmotic pressure; ○, haemolymph chloride concentration. Vertical lines indicate extent of standard deviations from mean values for haemolymph osmotic pressure of six larvae kept in tap water (see text). In six *L. stigma* larvae kept in tap water the mean haemolymph chlorine concentration was 11 ± 4·5 mM./l.

(*c*) *Haemolymph sodium*

In both species the normal concentration of sodium in the haemolymph is very similar to that found in other aquatic insects. The mean value for six *A. nervosa* larvae was 101 ± 3 mM./l. sodium.

In sea-water media the haemolymph sodium concentration increased and remained hypertonic to the medium (Fig. 2). Thus at an external concentration of 195 mM./l. sodium the concentration in the haemolymph of *L. stigma* larvae was twice the normal level. This is strikingly different from the regulation of haemolymph sodium in *L. affinis* larvae, in which the sodium was distinctly hypotonic at an external concentration of 195 mM./l., and was not increased to twice the normal level until the external sodium concentration reached 290 mM./l. (Sutcliffe, 1961).

(*d*) *Rectal fluid osmotic pressure and chloride*

The rectal fluid of tap-water larvae was considerably hypo-osmotic to the haemolymph, but when the concentration of the latter was raised by placing larvae in sea-water media the osmotic pressure of the rectal fluid increased rapidly to become

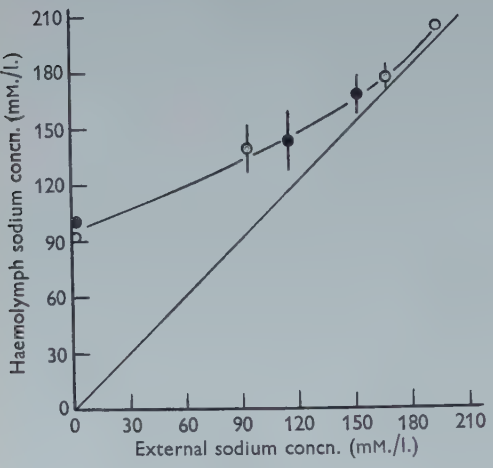


Fig. 2

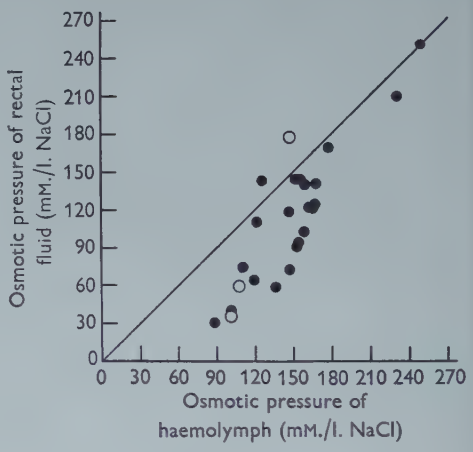
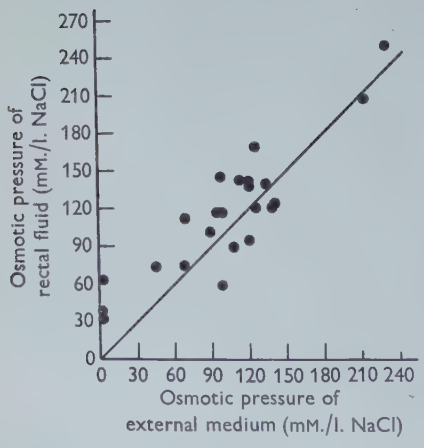


Fig. 3



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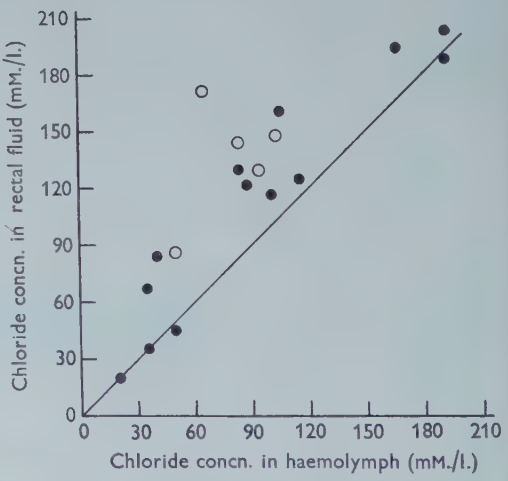

Fig. 5

Fig. 2. The relation between the external sodium concentration and the sodium concentration in haemolymph of *L. stigma* larvae (○) and *A. nervosa* larvae (●). Each point represents the mean value of haemolymph samples from five to six larvae (*A. nervosa*) and two to seven larvae (*L. stigma*). Vertical lines indicate extent of standard deviations of five or more haemolymph samples.

Fig. 3. Increase in the osmotic pressure of rectal fluid as the osmotic pressure of the haemolymph is raised. ●, Rectal fluid of *A. nervosa* larvae; ○, rectal fluid of *L. stigma* larvae.

Fig. 4 The relation between the osmotic pressure of sea-water media and the osmotic pressure of rectal fluid in *A. nervosa* larvae.

Fig. 5. Increase in the chloride concentration of rectal fluid as the concentration is raised in the haemolymph of *L. stigma* larvae (○) and *A. nervosa* larvae (●).

roughly iso-osmotic with the haemolymph (Fig. 3). In one instance in both *L. stigma* and *A. nervosa* the rectal fluid was hyper-osmotic to the haemolymph.

It is of some interest to note that in most instances the rectal fluid was slightly hyper-osmotic to the external medium (Fig. 4). Thus it is possible for the larva to drink salt water and excrete it as a slightly concentrated solution of salts, thereby gaining a small quantity of osmotically free water. In this respect it appears that the freshwater caddis larvae do not differ markedly from *L. affinis* larvae. Furthermore, as in *L. affinis*, the Malpighian tubule-rectal system in *L. stigma* and *A. nervosa* larvae can elaborate a fluid in which the chloride concentration exceeds that in the haemolymph (Fig. 5). This production of hypertonic rectal fluid is stimulated by the increase in haemolymph chloride which occurs when larvae are placed in sea-water media.

(*e*) *Osmotic pressure of the midgut fluid*

Samples of fluid were removed from the midgut and treated as described previously for *L. affinis* larvae. As in *L. affinis*, the midgut fluid in both *L. stigma* and *A. nervosa* contained a soluble dark brown pigment. The fluid was consistently hyper-osmotic

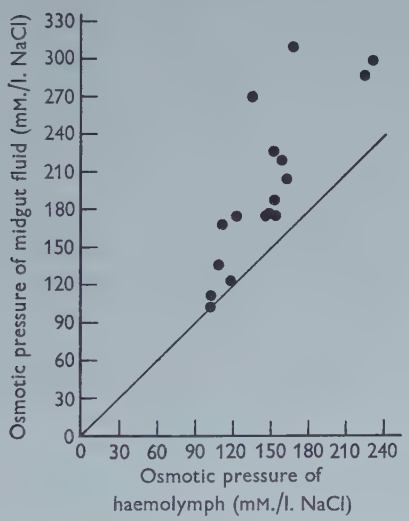

Fig. 6. The relation between the osmotic pressures of midgut fluid and haemolymph in *A. nervosa* larvae.

to the haemolymph, even when larvae were kept in tap water (Fig. 6). The significance of these findings cannot at present be explained, but it is worth noting that in mosquito larvae the midgut and caecal fluids are also hyper-osmotic to the haemolymph, associated with an internal circulation of water between the haemolymph and the gut (Ramsay, 1950).

(*f*) *Permeability of the body wall to sodium*

Using ^{24}Na as a tracer it was found that the body wall of *L. affinis* larvae is relatively impermeable to the inward diffusion of sodium, and that most of the sodium uptake occurs through the gut wall (Sutcliffe, 1961). Preliminary measurements of ^{24}Na influx in larvae of *L. stigma* and *A. nervosa* were also carried out as described for

L. affinis. Larvae were first adapted to tap water and to an external sodium concentration of 100 mM./l., and then some of the larvae were prevented from drinking the medium by sealing the mouth with a small blob of wax. After a further 2 days both media were replaced by a filtered sea-water medium containing 100 mM./l. sodium and a very small quantity of ^{24}Na. Now the influx of ^{24}Na should proceed exponentially to equilibrium with the outside concentration of labelled sodium, and the rate of influx will be proportional to the ratio C_i/C_o where C_i is the internal concentration and C_o is the external concentration of unlabelled sodium (Sutcliffe, 1961). In the case of larvae adapted to tap water before transference to the labelled solution containing 100 mM./l. sodium, the ratio C_i/C_o may be regarded as unity (see Fig. 2). For larvae previously adapted to an external concentration of 100 mM./l. sodium, C_i = 136 mM./l. sodium (Fig. 2).

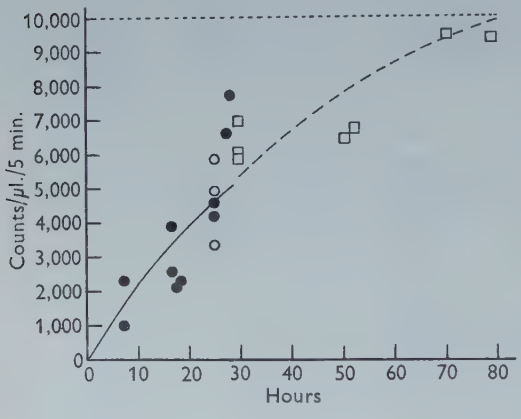

Fig. 7

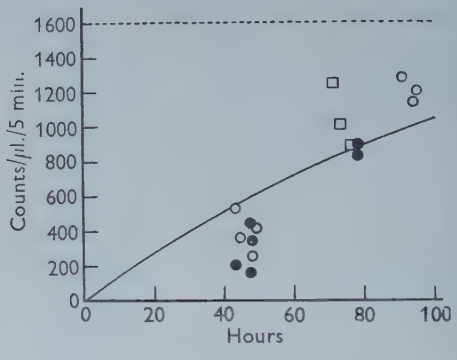

Fig. 8

Fig. 7. Influx of ^{24}Na into the haemolymph of *L. stigma* larvae in a sea-water medium containing 100 mM./l. sodium. □, Larvae previously adapted to the sea-water medium; ○, larvae transferred from tap water at the start of the experiment; ●, as above, but prevented from drinking the medium by sealing the mouths with wax. The two exponential curves describe theoretical increases in haemolymph radioactivity when T = 40 hr. and C_i/C_o = unity (solid curve), and when T = 60 hr. and C_i/C_o = 1·36 (broken curve). The horizontal broken line indicates the concentration of ^{24}Na in the sea-water medium.

Fig. 8. Influx of ^{24}Na into the haemolymph of *A. nervosa* larvae in a sea-water medium containing 100 mM./l. sodium. ○, Larvae previously adapted to the sea-water medium; ●, as above, but prevented from drinking the medium by sealing the mouths with wax; □, larvae transferred from tap water at the start of the experiment. The exponential curve describes the theoretical increase in haemolymph radioactivity when T = 150 hr. and C_i/C_o = 1·36. The horizontal broken line indicates the concentration of ^{24}Na in the sea-water medium.

The results of radioactivity counts on haemolymph samples from individual *L. stigma* larvae are shown in Fig. 7, together with two theoretical curves describing exponential increases in haemolymph radioactivity. The results obtained on larvae previously adapted to tap water conform approximately to a curve where T = 40 hr. and C_i/C_o = unity. Results obtained on larvae previously adapted to an external concentration of 100 mM./l. sodium conform to a curve where T = 60 hr. and C_i/C_o = 1·36. Two interesting points emerge clearly from a study of Fig. 7; (*a*) the

rate of ^{24}Na influx is very high, and (*b*) the rate of influx through the body wall is so fast that it obscures the influx through the mouth and gut wall, in spite of the fact that larvae not prevented from drinking were swallowing large quantities of the labelled sea-water medium (evidence of drinking will be given in a following paper).

The rate of influx through the body wall of *L. stigma* larvae (T = 40 hr.) may be compared with that in *L. affinis* larvae at an external concentration of 100 mM./l. sodium, where T = 400 hr. (Sutcliffe, 1961). Thus it appears that the influx rate in *L. stigma* larvae is ten times that in *L. affinis* larvae. Similar results were obtained with larvae of *A. nervosa* (Fig. 8). Here, the results may be compared with an exponential curve where T = 150 hr. and C_i/C_o = 1·36. Hence the rate of influx through the body wall is two to three times greater than in *L. affinis* larvae. Now if the rate of ^{24}Na exchange through the body wall is also a measure of the rate of diffusion of unlabelled sodium, it would follow that the body wall in the freshwater caddises is considerably more permeable to sodium than the body wall of the euryhaline larvae of *L. affinis*. In the case of *L. affinis* larvae the preceding assumption is not unreasonable, as the rate of ^{24}Na influx through the body wall is roughly proportional to the external concentration of unlabelled sodium (Sutcliffe, 1961). It is therefore possible that the rate of ^{24}Na influx in the freshwater caddises is also a measure of unlabelled sodium diffusion. However, the results here presented are also open to other interpretations. Thus the possibility is not excluded that either active uptake of sodium, or exchange diffusion, or a combination of both phenomena is responsible for the higher rate of ^{24}Na influx in the freshwater caddises. The interpretation of these differences in influx rates must therefore wait until the nature of the sodium exchange mechanism in these caddis larvae has been studied in more detail. Nevertheless, it appears that there is a distinct difference between the freshwater caddises and *L. affinis* with respect to the exchange of labelled sodium through the body wall, and it is suggested that this difference is an adaptive feature associated with the maintenance of a low sodium concentration in the haemolymph of *L. affinis* larvae.

DISCUSSION

The range of external salt concentrations tolerated by larvae of *L. stigma* and *A. nervosa* is strikingly different from that tolerated by larvae of *L. affinis*. The latter live for months at an external concentration of 410 mM./l. NaCl, and tolerate even higher salt concentrations for several days (Sutcliffe, 1960, 1961). Larvae of *L. stigma* and *A. nervosa*, however, begin to die at external concentrations greater than about 60 mM./l. NaCl, and only a few individuals survived for about one week at external concentrations of 170–220 mM./l. NaCl. This contrast in salt tolerance is accompanied by marked differences in regulation of the haemolymph composition. In the freshwater caddises the total concentration of the haemolymph is almost entirely accounted for by the cation sodium, and in salt water the haemolymph sodium level increases to remain hypertonic to the external medium. On the other hand, in *L. affinis* the haemolymph sodium concentration of larvae kept in fresh water accounts for only some 80% of the total haemolymph concentration, and the remaining 20% is due to non-electrolytes (Sutcliffe, 1961). Furthermore, in *L. affinis* the haemolymph sodium level is maintained strongly hypotonic to high external salt concentrations, and the

concentration of the non-electrolyte fraction in the haemolymph is greatly increased. The haemolymph chloride level in *L. affinis* is also maintained strongly hypotonic to high external salt concentrations. In the freshwater caddises, however, although haemolymph chloride is maintained hypotonic to salt water, the chloride concentration steadily increases as the external concentration is raised, and it eventually contributes most of the total haemolymph concentration on the anion side.

So far as chloride is concerned, it appears that the concentration in the haemolymph is to some extent actively regulated by the Malpighian tubule-rectal system. This system is very sensitive to changes in the haemolymph chloride level. An increase in the latter is rapidly followed by a considerable increase in the chloride concentration of the rectal fluid, which can be slightly hypertonic to the concentration in the haemolymph. In *L. affinis* larvae the ability to regulate the haemolymph chloride level is greatly increased, and the chloride concentration in the rectal fluid can exceed that in the haemolymph by a factor of three (Sutcliffe, 1961). This must be regarded as an adaptive feature for survival at high external salt concentrations.

The ability of caddis larvae to elaborate an excretory fluid in which the chloride concentration exceeds that in the haemolymph was first demonstrated in *Limnephilus flavicornis* by Boné & Koch (1942), and is now firmly established as a characteristic feature of the Limnephilidae. Boné & Koch also showed that a reduction in the haemolymph chloride concentration of *L. flavicornis* larvae resulted in re-absorption of chloride from the fluid in the rectum. Thus the Malpighian tubule-rectal system regulates the haemolymph chloride level over a fairly wide range of external concentrations. This is strikingly different from the behaviour of *Sialis* larvae, which are unable to produce an excretory fluid hypertonic with respect to the haemolymph chloride concentration (Shaw, 1955). In *Sialis* the excretory system is slow to respond to changes in the haemolymph chloride level, which remains hypertonic over a range of external concentrations up to at least 120 mM./l. NaCl. In view of this difference between caddis larvae and *Sialis*, it seems likely that the excretory system in freshwater mosquito larvae (Wigglesworth, 1938) and *Helodes* larvae (Treherne, 1954) can also concentrate chloride from the haemolymph, since these insects also maintain the haemolymph chloride hypotonic to salt water. It is certainly probable that the salt-water larvae of *Aedes detritus* are able to concentrate chloride, as these larvae drink salt water and produce a rectal fluid considerably hyper-osmotic to the haemolymph (Ramsay, 1950).

In maintaining a haemolymph sodium level slightly hypertonic to high external concentrations the larvae of *L. stigma* and *A. nervosa* closely resemble the larvae of *Aëdes aegypti* (Ramsay, 1951, 1953) and *Sialis* (Shaw, 1955). Since neither of these latter insects can elaborate an excretory fluid hypertonic with respect to the haemolymph sodium concentration, it would be interesting to investigate sodium output in the rectal fluid of the caddis larvae. It seems *a priori* likely that the freshwater caddises are also unable to concentrate sodium. It is probable, however, that the euryhaline larvae of *L. affinis* can elaborate rectal fluid in which the sodium concentration exceeds that in the haemolymph. Indeed, if the Malpighian tubule-rectal system is the sole route for salt excretion, it may be argued that the ability to concentrate sodium is necessary in larvae which drink salt water with a sodium concentration greater than that in the haemolymph, and yet maintain the haemolymph sodium level

strongly hypotonic. Moreover, since the rectal fluid in *L. affinis* larvae consists of a highly concentrated solution of chloride, it is reasonable to suppose that this is balanced by a high concentration of sodium ions.

In the preceding paper it was suggested that maintenance of a low haemolymph salt concentration in *L. affinis* larvae is concerned with the regulation of a low salt concentration in the tissue cells. Shaw (1959) has suggested that the lethal effect of salt water on the freshwater crab *Potamon niloticus* is due to the action of the raised blood concentration on muscle fibres, and that part of this action is the increased penetration of sodium ions into the interior of the fibre. It is possible that a similar penetration of sodium into tissue cells occurs following an increase in the haemolymph sodium level in caddis larvae. If this is so, the active maintenance of a low haemolymph sodium level in *L. affinis* would be extremely important, and would form a major part of the adaptation for survival at high external salt concentrations. It is perhaps significant that *L. affinis* larvae do not survive for more than a few days when the external sodium concentration is raised to about 425 mM./l. At this concentration, regulation of a low sodium level in the haemolymph breaks down (Sutcliffe, 1961). In the freshwater caddises the haemolymph sodium level is not regulated, and larvae die rapidly when the sodium concentration in the haemolymph is raised to about twice the normal level.

The maintenance of a low haemolymph sodium level in *L. affinis* larvae may also be due to adaptive features situated outside the excretory system. One of these features may be a reduction in permeability of the body wall to sodium compared with that in the freshwater caddises. A reduction in permeability to sodium (and to chloride) would confer a considerable advantage, in that the salt load carried by the excretory system in order to eliminate excess salts gained by diffusion through the cuticle will be decreased. Consequently, the capacity of the excretory system to deal with excess salts gained by drinking salt water will be correspondingly increased. Combined with the ability to produce a concentrated excretory fluid, this feature alone could, to a certain extent, increase the tolerance of *L. affinis* larvae to salt water.

SUMMARY

1. Survival and regulation in sea-water media was studied in the freshwater caddises *Limnephilus stigma* and *Anabolia nervosa*.

2. The majority of larvae did not survive for more than a few days at external salt concentrations greater than about 60 mM./l. NaCl.

3. In sea-water media the haemolymph osmotic pressure increased to remain slightly hyper-osmotic to the medium. The haemolymph sodium level also increased to remain slightly hypertonic to the medium, but the chloride level was maintained hypotonic until just prior to death of the larvae.

4. When the haemolymph chloride concentration was raised above the normal level, the Malpighian tubule-rectal system elaborated fluid in which the chloride concentration was hypertonic to the haemolymph. The system is highly sensitive to changes in the haemolymph chloride level.

5. The regulation of body-fluid composition in the freshwater caddises is compared with that found previously in the euryhaline larvae of *Limnephilus affinis*. It is

suggested that the maintenance of a low haemolymph sodium concentration in *L. affinis* larvae is an important part of the adaptation for survival in salt water.

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THE RESPONSE OF THE DOGFISH TO ANOXIA

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It has been suggested repeatedly that the mammalian response to anoxia of hyperpnoea and hypertension may be the survival in air-breathing vertebrates of a phylogenetically primitive mechanism present in fish (Marshall & Rosenfeld, 1936; Schmidt & Comroe, 1940; Schmidt, 1956; Heymans & Neil, 1958). Black (1951), reviewing the results of four previous studies on the effect of deoxygenated sea water on elasmobranch fish, concluded that there was no change in respiration, whilst Lutz (1930) showed that restriction of water flow through the dogfish pharynx (a change likely to cause anoxia) resulted in a slowing both of respiration and heart beat. Thus the evidence, fragmentary though it is, does not suggest that the response of an elasmobranch fish to anoxia is like that of a mammal. In an attempt to re-examine this problem using the common local dogfish *Squalus acanthias** L., the work of Lutz has been confirmed and amplified. Reduction of the minute volume of pharyngeal flow caused bradycardia and respiratory slowing. It soon became clear, however, that the responses of the heart and of respiration were mediated by separate mechanisms. Respiratory rate was related primarily to minute volume and was much less influenced by the oxygen content of the water respired. The heart, by contrast, was reflexly slowed by anoxia, whether this was induced by a restricted flow of normally oxygenated water or an ample flow of deoxygenated water.

In this paper the influence of changes in minute volume on the cardiac and respiratory rates will be re-examined and the mechanisms mediating the response to anoxia will be described. The reflexes concerned in the response of respiration to changing flow rate and the receptors from which they arise will be presented in a subsequent paper.

MATERIAL AND METHODS

Mounting and perfusion

Thirty-three specimens of *S. acanthias* varying from $3\frac{1}{2}$ to 6 lb. in weight were used. Each fish was secured by two clamps to the trunk and one to the snout in a rectangular tank filled with cooled circulating sea water; the water temperature varied from 8° to 15° C. If the fish was to be dissected, it was anaesthetized by an injection of 3 ml. of 1% MS222 Sandoz.

In all experiments except one the mouth was sewn closed and the spiracles were cannulated with specially shaped glass tubes which fitted snugly inside the spiracular rim. These cannulae were connected to a receiving vessel into which sea water poured at a known rate. This rate was changed by a series of resistances that could be cut in

* I am indebted to Dr J. Garrick, of the Dominion Museum, Wellington, for pointing out that *Squalus lebruni* Vaillant, the name previously used by me for this species, is a synonym of *S. acanthias* L.

and out of circuit by means of taps, providing sixteen known flow rates varying from zero to 985 ml./min. Deoxygenated or CO_2-enriched sea water was supplied to the fish by a two-way tap inserted close to the cannula junction. Similar flow rates of normal and deoxygenated water were achieved by ensuring that the polythene connexions between the two-way tap and the two supplies of water were identical in length and diameter, and that the pressure heads were identical. As the deoxygenated water left its container, the space above was filled with nitrogen, maintained at atmospheric pressure with the aid of a manometer.

Deoxygenated sea water was prepared by boiling the water for at least 2 hr. at 160 mm. Hg and bubbling with nitrogen for 2 hr. when cool. CO_2-enriched sea water was prepared by equilibrating sea water with 'Carbogen' (5% CO_2, 95% O_2) and returning the pH to 7·9 (the pH of local sea water) with sodium bicarbonate. Normal and deoxygenated sea water were maintained at temperatures that did not differ by more than 2° C., and results were not accepted until they had been demonstrated with the deoxygenated water both above and below the temperature of the normal sea water.

Recording

Records were made on either an Ediswan or a Both four-channel pen writer. The electrocardiogram (e.c.g.) was recorded in the manner described in a previous paper (Satchell, 1961); respiratory movement was recorded using a Statham strain gauge and amplifier. The strain gauge was attached by a thread either to the side of the pharynx above the first gill opening, or, if the fish were mounted upside down, to the region of the mandibular symphysis. The output of the strain gauge was amplified by an a.c.-coupled amplifier (time constant 1 sec.) in earlier experiments; by a d.c.-coupled amplifier in later experiments.

Blood pressure was recorded using the sensitive Statham P23BB pressure transducer coupled by 61 mm. of 2 mm. P.V.C. tubing to a no. 19 needle 1 in. long inserted into the dorsal aorta. This was exposed by cutting off the tail at the level of the posterior dorsal fin. The P23BB transducer does not have a good high-frequency response (9 cyc./sec. with critical damping) but in these experiments it was the systolic and diastolic pressures that were of interest, rather than the wave-form of individual pulse beats. The transducer output was amplified by a carrier-wave amplifier, and calibrated repeatedly during the experiment by recording known pressures; all pressures are given in mm. Hg above the tank water level.

RESULTS

The relation of minute volume to respiratory and cardiac rate

A curve showing the respiratory and heart rates at sixteen different minute volumes ranging from zero (flow turned off) to 985 ml./min. is shown in Fig. 1. Each point is an average of the heart beats or respirations occurring during 1 min. Low rates of flow were not allowed to continue for periods longer than this, and were interspersed with periods of maximum flow.

The respiratory rate (Fig. 1B, ●) decreased with decreasing minut evolume throughout the range. The more pronounced slowing as the flow rate dropped below 60 ml./min. was a constant feature; as the respiration slowed, its amplitude increased.

Visual observation showed the fish to be making greater inspiratory efforts, and at rates below 200 ml./min. the closed external gill openings became more obviously indented during the inspiratory phase. Schoenlein & Willem (1895) noted this in the skate and described such respiration as dyspnoeic.

The curve of heart rate (Fig. 1A, ●) tended to flatten out at both low and high flow rates. Though the resting rate varied from one fish to another, the shape of the curve, with the increased steepening at rates below 200 ml./min., was seen in other experiments.

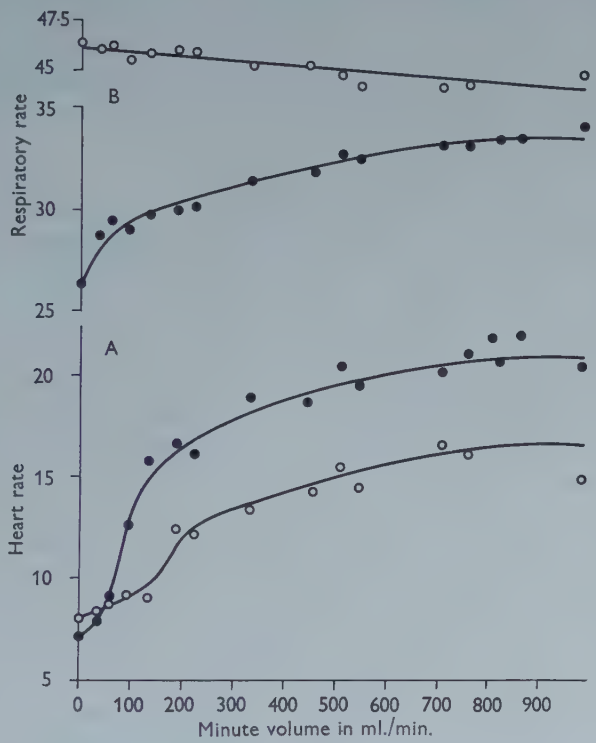

Fig. 1. A, cardiac and B, respiratory rates at different minute volumes of pharyngeal water flow. ●, Normal, 8° C.; ○, curarized, 9° C.

Is the slowing of heart and respiration due to myocardial or medullary anoxia?

Reducing the flow of water through the pharynx usually caused a cough, and this is itself liable to cause cardiac slowing. But even when no cough occurred, the next heart beat following the reduction in flow rate was delayed. The short latency of this response (3–4 sec. depending on the heart rate) argues against a direct action of anoxia on the myocardium or medulla.

Intravenous atropin (0·01 mg./kg.) blocked the action of the cardiac vagus and abolished the short-latency slowing of the heart. When the water flow was stopped no cardiac slowing appeared until 5 min. had elapsed, and even after 12 min. the rate was still twice that observed with zero flow before atropinization. Cutting the cardiac vagi similarly abolished the short-latency cardiac response to decreased minute volume.

The muscles effecting closure of the gill slits have proved to be more resistant to the

action of curare than the rest of the branchial musculature, and 0·075 mg./kg. of tubocurarine produced a preparation in which all respiratory movement appeared to have ceased. Re-attaching the thread of the strain gauge to the anterior margin of the first gill flap and increasing the gain of the amplifier showed, however, that some muscle fibres remained active, for a recordable trace resulted. Following curarization to this extent respiratory rate and minute volume ceased to be related. The significance of this finding will be discussed in a subsequent paper, but the observation that the respiratory rate had not fallen after 5 min. of zero flow is relevant here. With continued zero flow the respiratory rate of 26/min. recorded for zero flow before curarization was not achieved until the eighth minute. Thus the evidence indicates that although myocardial and central anoxia do produce cardiac and respiratory slowing if the water flow is turned off for more than 5 min., they cannot explain the short-latency slowing in response to reduced flow.

Is the slowing of heart and respiration mediated by a common mechanism?

Evidence on this was obtained from experiments in which deoxygenated sea water could be switched into the perfusion circuit. The normal slowing of heart and respiration in response to a reduced or zero flow of normally oxygenated sea water was first recorded; then a flow of deoxygenated sea water was turned on. The respiration promptly speeded up again, and the same range of respiratory rates as had previously been witnessed with different minute volumes of normally oxygenated sea water could be demonstrated by varying the flow rate of deoxygenated sea water. A low or zero flow of deoxygenated sea water produced respiratory slowing: a faster rate caused the respiration to speed up.

The behaviour of the heart contrasted with that of respiration. A flow of deoxygenated sea water caused a slight enhancement of the slowing already resulting from a zero flow of normally oxygenated sea water: alteration of the flow rate of deoxygenated sea water did not change the bradycardia, which persisted as long as deoxygenated sea water remained in the pharynx. Restoration of normally oxygenated water caused the heart to speed once again within one or two beats of the change.

The results suggest that the respiratory response is dependent on flow whether it be of oxygenated or deoxygenated water, whilst the cardiac response is not primarily related to flow, but to the rate at which oxygen is supplied to the fish.

The responses of the fish curarized as described in the previous section are significant here and are shown in Fig. 1. The respiratory rate (Fig. 1 B, ○) showed no significant changes with alteration in minute volume; statistically the two are unrelated ($r = -0{\cdot}27$, $P > 0{\cdot}10$). Changes in the amplitude of respiration occurred, but in the absence of normal movement failed to evoke the corresponding changes in rate. The heart, in contrast, still slowed in response to a reduction in minute volume (Fig. 1 A, ○), showing that this mechanism was independent of respiratory movement. Similarly, deoxygenated water caused cardiac slowing in the curarized fish.

At this stage in the study it became clear that since respiratory rate was so dependent on the rate of water flow regardless of its oxygen content, any effect that anoxia might have on respiration could only be evaluated if precautions were taken to ensure that the flow rates of normal and deoxygenated sea water were identical. All data presented subsequently in this paper were obtained from experiments in which this was done.

Changes in heart rate and blood pressure in response to anoxia

The period of deoxygenated water flow was set routinely at 2 min. Myocardial anoxia was not likely to be causing slowing of the heart rate since in an atropinized fish the rate showed no change within 2 min., and even after 5 min. flow of deoxygenated water had only fallen from 38 to 35/min. Flow rates of deoxygenated and normally oxygenated water were always kept the same, and varied in different experiments from 1 l./min. to 500 ml./min.

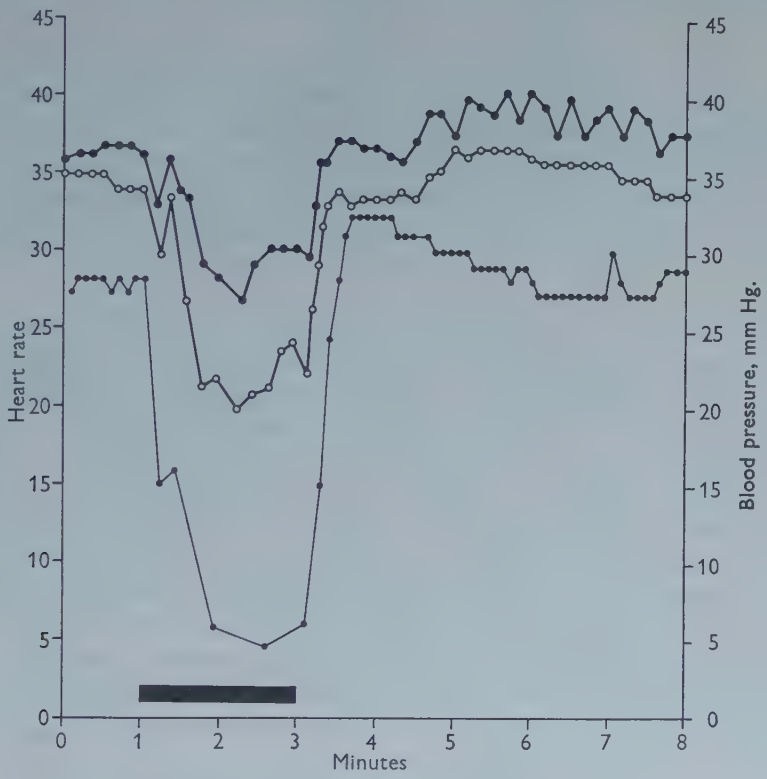

Fig. 2. Change in heart rate (lower curve), and in diastolic (middle curve, ○) and systolic (upper curve, ●) blood pressure, caused by a 2 min. flow of deoxygenated water = black inset. 12° C.

The change in heart rate is shown in Fig. 2. Each rate determination is an average derived from the three preceding beats. The sudden onset and cessation of the slowing is evident. The restoration of normally oxygenated water was always followed by an acceleration to a level above the resting rate, which was not regained until the end of the third minute after the return of normal water. This pattern of change was very constant from one experiment to another, the slowest rate obtaining during the deoxygenated water flow being between 5 and 7/min. The tendency for the slowing to be incompletely maintained and for the rate to rise towards the end of the second minute was a general feature not well shown in Fig. 2, but apparent in Fig. 3A, ●.

Blood-pressure records from the dorsal aorta showed a drop in systolic pressure

(Fig. 2, ●) of 10 mm. Hg from the resting systolic level of 36 mm. Hg. The diastolic pressure (Fig. 2, ○) fell 14 mm. from its resting level of 34 mm. Hg. The increase in the pulse pressure from 2·3 to 6 mm. Hg was due to the low diastolic pressure resulting from the less frequent heart beats. Following the return of normally oxygenated water the systolic pressure rose to 40 mm. Hg and this hypertensive overshoot outlasted the period of raised heart rate, suggesting some increase in resistance in the peripheral circulation. The saw-toothed contour of the record of systolic pressure during this period was due to the increase in blood pressure at each expiration periodically coinciding with the systolic peak of pressure.

Respiratory changes in response to anoxia

The changes in respiratory rate evoked by deoxygenated water were not profound; Fig. 3B, ●, is typical. During the first minute the rate fell from 26·5 to 23·5/min., and then rose again to exceed the resting rate, attaining 29·5/min. in the second minute. With the return of normally oxygenated water the rate did not immediately fall; in some fish a further increase in rate occurred. Sometimes the normal rate was re-established within 2 min.; more commonly it slowly returned to the resting rate over 10 min.

The depth of respiration increased during the period of deoxygenated water flow, sometimes steadily throughout the 2 min., sometimes only during the first minute. The changes in depth were never very marked and in some experiments were scarcely discernible. Compared with the changes in depth that could be evoked by varying the flow rate, they were small indeed.

The possibility that closing the mouth and cannulating the spiracles itself masked some respiratory or cardiac response was excluded in an experiment in which the head and fore part of the body were enclosed in a separate container within the main tank. Deoxygenated or normal water was fed into this container, the fish respiring freely with its mouth and spiracles unimpeded. Inevitably the change from normal to deoxygenated sea water was slow and incomplete but the characteristic respiratory responses of decreased rate and increased depth were seen. Cardiac slowing, though less profound, was quite evident.

The mechanisms mediating the response to anoxia

It is now generally agreed that the respiratory hyperpnoea of mammals in response to anoxia is reflexly mediated and originates in the chemoreceptor cells in the carotid and aortic bodies (Adams, 1958; Heymans & Neil, 1958).

Granel (1927), in a detailed morphological study of the pseudobranch in fish, showed that in teleosts there are clusters of acidophil cells surrounding the blood vessels which resemble the glomus tissue and chemoreceptor cells of the carotid body in mammals. The possibility that the response to anoxia in the dogfish depended on receptors in the pseudobranch had therefore to be examined. Experiments failed to confirm this. Fish in which both pseudobranchs were (*a*) surgically removed, (*b*) extensively cauterized, (*c*) frozen by cannulating the spiracles with copper tubes conducting freezing mixture at -5° C., (*d*) rendered ischaemic by cutting or clipping the afferent pseudobranchial artery, all showed an unimpaired response to anoxia.

However, in experiments involving gill deafferentation the pseudobranchs were routinely cauterized in case some small part of the response to anoxia depended on them.

The gills were deafferented by opening the anterior cardinal sinus and cutting the glossopharyngeal, the first three branches of the vagal and the pretrematic branch of the fourth vagal branchial nerves. The post-trematic branch of the fourth vagal branchial nerve carries inhibitory fibres to the heart and was left intact; the deafferentation was not thereby impaired since there is no gill on the posterior wall of the fifth gill pouch.

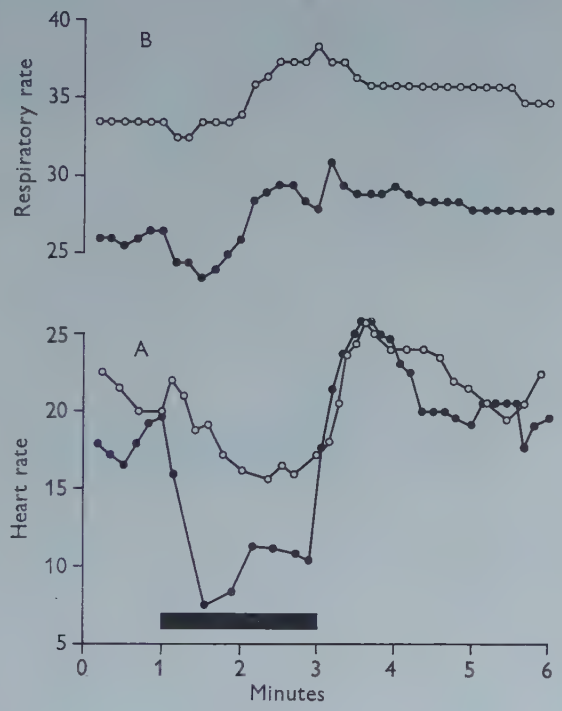

Fig. 3. Changes in A, cardiac and B respiratory rate caused by a 2 min. flow of deoxygenated water = black inset. ●, normal, 10–11° C. ○, after gill deafferentation. 14–15° C.

In Fig. 3A the cardiac responses to anoxia before, ●, and after, ○, deafferentation are compared. Following deafferentation, the bradycardia during the period of deoxygenated water flow was less intense, and its onset was delayed, 90 sec. elapsing before slowing was fully developed; the speeding of the heart following the return of normally oxygenated water was not significantly changed. The persistence of some cardiac response to anoxia, although of reduced intensity and delayed onset, could be interpreted in one of two ways. *Either* the response to anoxia was confined to the central nervous system, being dependent on the flow of deoxygenated blood from the gills to the brain. Its impairment might then be ascribed to the blood loss and injury incidental to deafferentation. *Or* there existed both a peripheral reflex response characterized by its intensity and speed of onset, and a central direct response dependent on the attainment of a critical level of anoxia in the brain and therefore of longer latency.

The first interpretation was excluded by experiments in which the conus arteriosus was exposed and clamped with artery forceps. After the lapse of 30 sec., to allow the blood in the ventral aorta to pass into the peripheral circulation, deoxygenated water was turned on. The heart was inhibited with undiminished intensity (Fig. 4, ○) and the return of normally oxygenated water caused the heart to speed again. This was significant in excluding the possibility that anoxia of central origin could alone account for the slowing. As will be shown later, such slowing does occur but it is less intense

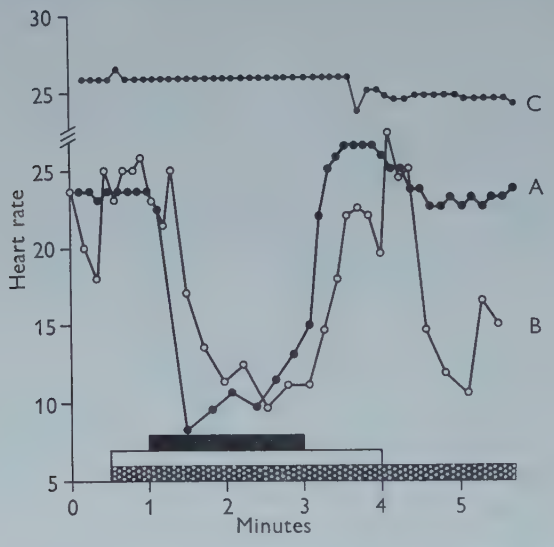

Fig. 4. Changes in heart rate: A, caused by a 2 min. flow of deoxygenated water (black inset); B, the same but with conus occluded (white inset). C, changes caused by conus occlusion alone (stippled inset) after atropinization. 10–11° C.

and has a longer latency. Following the removal of the artery forceps there was a second slowing as the banked-up venous blood passed rapidly into the branchial vessels. In a further experiment the conus was clamped and the ventral aorta cut at the junction of the conus with the aorta. Ringer's fluid was perfused into the dorsal aorta so that it welled out of the cut end of the ventral aorta. Despite this retrograde perfusion of the branchial blood vessels deoxygenated water still caused cardiac slowing and the heart accelerated on the return of normally oxygenated water. This evidence points strongly to the existence of a reflex response to anoxia with the gills as the most likely source of the afferent information in that they are thin walled and the most permeable structures in the path of the deoxygenated water.

The cardiac response that persisted after deafferentation was presumed to originate in the brain. Evidence supporting this has been obtained in experiments on de-afferented fish perfused with normally oxygenated sea water in which the conus has been clamped with artery forceps for a 4 min. period. In Fig. 5 the response is compared with that evoked by a 2 min. period of deoxygenated water flow without the clamp. Despite the disturbances in heart rate associated with closing the artery forceps, the heart slowed steadily from the end of the second minute to the removal of the clamp. In the atropinized fish (Fig. 4) the heart had only slowed from 26·5 to 24/min.

after 6 min. of conus occlusion. The intense slowing that occurred after removing the artery forceps was, as in the intact fish, associated with the surge of banked-up venous blood through the gills to the brain. It occurred in other experiments in which the conus was clamped for 6 or 8 min. and was associated with removal of the forceps rather than with a particular duration of conus occlusion. As it occurred in a deafferented fish, it must also be related to a central response to inadequately oxygenated blood.

When the two traces of Fig. 5 are compared, it is seen that in a deafferented fish a 2 min. flow of deoxygenated water evoked cardiac slowing sooner (50 sec.) than did occluding the conus (2 min.). Presumably perfusion of the brain with blood equilibrated with deoxygenated water lowered the oxygen tension more rapidly and more completely than did the stasis imposed by occluding the conus.

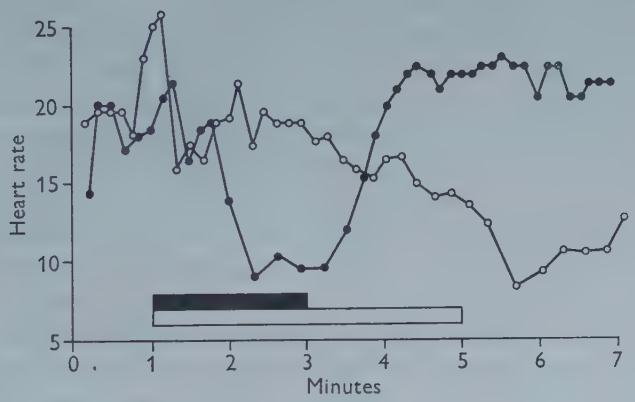

Fig. 5. Changes in cardiac rate in fish with gills deafferented caused by: ●, 2 min. flow of deoxygenated water (black inset); ○, a 4 min. period of conus occlusion (white inset). 14–15° C.

The speeding of the heart following the period of anoxia

As the cardiac acceleration beyond the original rate, following the return of normal water flow, persisted in the deafferented fish (Fig. 3A), it may be ascribed to some change either in the brain or in the pacemaker of the heart itself. The slowing of the heart and reduction in blood flow during anoxia must result in an increase in carbon dioxide tension and in hydrogen-ion concentration in the medulla and peripheral tissues alike. The response to perfusion of the pharynx with water equilibrated with 5% CO_2 and 95% O_2 was investigated. In the intact fish a profound cardiac and respiratory inhibition resulted. Little significance is attached to this, since such a concentration of CO_2 may well be an irritant to the gills. However, in the deafferented fish 5% CO_2 caused acceleration of heart rate from 19 to 25/min. and of respiration from 46 to 48/min., without any early inhibitory episode. Thus a raised tension of CO_2 in the brain and tissues caused cardiac acceleration and may account for the speeding following anoxia. It is not possible at present to decide whether the effect is exerted upon the vagal centres or upon the cardiac pacemaker. As the respiratory rate is simultaneously changed in the same direction, it is likely that there is a central response.

Respiratory responses in the deafferented fish

Deafferentation eliminated the respiratory slowing (Fig. 3B, ○) and increase in depth associated with deoxygenated water, but the subsequent acceleration remained. The evidence cited above that 5% CO_2 caused some respiratory speeding in the deafferented fish suggests that the speeding following deoxygenated water flow may similarly be ascribed to a rise in CO_2 tension in the brain. The slowing and the increase in the amplitude both appear to be reflex in origin.

DISCUSSION

The studies of Schoenlein & Willem (1895) on *Scyllium canicula* and *Torpedo ocellata*, of Bethe (1925) on *S. canicula* and *S. catulus* and of Ogden (1945) on *Mustelus antarcticus* agree that deoxygenated water has no effect on either rate or depth of respiration when captive fish are immersed in it. Their findings are not so at variance with those reported here, for the method of perfusion described earlier permitted high rates of deoxygenated water through the pharynx free of admixture with normal sea water. Notwithstanding that perfusion with deoxygenated water must have provided a maximal anoxic stimulus the changes in respiratory rate and depth were not profound. It is not surprising that they were unobserved by earlier workers; it may be doubted whether such changes, as distinct from the changes evoked by varying flow rates, ever occur naturally in these inhabitants of shallow, well-aerated seas.

Evidence has been presented that the cardiac slowing in response to decreased minute volume of water flowing through the pharynx was mediated by a cardio-inhibitory response to anoxia. This consisted of an intense reflex inhibition of short latency and a less powerful direct response of slower onset. The evidence from deafferentation suggests that the chemoreceptors are located in the gills; as the receptors responded to deoxygenated water in the absence of branchial blood flow it would appear that they were so superficially placed as to be accessible by diffusion from the gill epithelium. Apart from this, nothing is yet known of their structure, location or function.

The cardiac response to anoxia in mammals contrasts with that in fish; anoxia accelerates the mammalian heart and elevates the blood pressure. Conflicting results have come from the injection of drugs such as cyanide and nicotine into the carotid body; some workers have reported bradycardia to be caused by stimulating the chemoreceptors in this way (Heymans, Bouckaert & Dautrebande, 1931; Heymans & Bouckaert, 1941). More recently, Daly & Scott (1958) have shown, in the dog, that when the hyperpnoea of anoxia is prevented by artificial positive pressure ventilation, perfusion of the carotid body with venous blood from a donor dog causes bradycardia. Moreover, in dogs spontaneously breathing room air, denervation of the lungs converts the cardiac acceleration, which is the normal response to such a perfusion, to a bradycardia, despite the hyperpnoea. They suggest that the cardio-acceleratory response to anoxia in mammals is a vagally relayed reflex from the lungs depending on hyperpnoea and masking an underlying bradycardia. The response of the dogfish may thus be regarded as the basic vertebrate response to anoxia, which in mammals is overridden

by a phylogenetically more recent reflex related to aerial respiration. Such a conclusion is opposed to the prevailing assumptions outlined in the introduction to this paper.

Any attempt to evaluate the significance of this bradycardia is hindered by the dearth of quantitative data on haemo-respiratory systems of fish. The following suggestions are offered only as an indication of the direction in which, it is hoped, further work will proceed. Krogh (1941) has emphasized that the large mass and low oxygen content of water as compared with air impose physical limitations on the extent to which the volume of oxygen transported to the gills can be increased. Despite the existence of reflexes, as yet incompletely known, whereby the magnitude of the inspiratory effort is inversely related to the volume of water in the pharynx, this limitation in aquatic respiration must exist. Moreover, the easier pharyngeal filling during forward swimming, and the temporary interference with water-intake occasioned by food prehension and swallowing, presumably must alter pharyngeal flow. The haemo-respiratory apparatus can be regarded as a mechanism by which the oxygen in solution in the incoming water is transferred to combine with haemoglobin in an outflowing stream of blood. The slowing of the heart in response to a reduction in pharyngeal flow, it is suggested, serves to regulate the cardiac output, so that the quantity of blood leaving the gills is no more than is necessary to permit the haemoglobin in it to be saturated by the oxygen obtainable from the amount of water presented to the gills at that time. Dill, Edwards & Florkin (1932) have shown that the blood leaving the gills of a skate is 93% saturated, closely approximating to that of man (95%). It is reasonable to infer that the cardiac slowing and fall of blood pressure do indicate a fall in cardiac output, since no system of vasomotor control has been reported in fish, and the blood-pressure changes do not occur in atropinized fish. More debatable is the implication that changes in the minute volume of flow through the pharynx would result in changes in saturation of the blood leaving the gills if the cardiac output remained unchanged. If the amount of water pumped through the gills were much in excess of what was necessary to oxygenate the blood there would be a margin of safety that would make such a regulation of cardiac output superfluous. But the high coefficient of utilization of oxygen in fish (50–80%, van Dam, 1938; Hazelhoff & Evenhuis, 1952) suggests that this is not so, and that a reduction in the minute volume of water without a corresponding change in cardiac output would result in a diminished saturation. The only way in which this could be avoided would be by an increase in the coefficient of utilization, and this clearly has an upper limit. Moreover, the high cost in pumping work done per unit of oxygen acquired makes it, *a priori*, unlikely that fish normally operate much below the maximum efficiency that the respiratory system permits.

These speculations may have relevance to the well established phenomenon of respiratory dependence in fish, reviewed by Fry (1957). In teleost fish active metabolism, as opposed to vital processes, decreases as the oxygen content of the water is reduced. Ferguson (1957) has published graphs relating the swimming speed of perch to the oxygen content of their water; as this falls their activity, as indicated by swimming speed, decreases. The response occurs before tensions low enough to affect the oxygen saturation of their haemoglobin are reached; its mechanism is unknown. If it is that a decrease in oxygen uptake by the gills reflexly results in a reduction both in

cardiac output and in motor activity, a mechanism whereby the needs of the tissues for oxygen are attuned to the ability of the respiratory system to supply it is evident. Such a mechanism would be very different from that in air-breathing vertebrates, yet appropriate to the physical limitations that aquatic respiration imposes.

SUMMARY

1. Curves relating the cardiac and respiratory rates of *Squalus acanthias* L. to the minute volume of water passing through the pharynx are presented; decreased minute volume caused respiratory and cardiac slowing.

2. The change in heart rate was dependent on anoxia; the respiratory response was largely independent of the oxygen content of the inspired water.

3. The respiratory and cardiac changes caused by deoxygenated water flow are described. Anoxia produced cardiac inhibition and a fall in blood pressure; respiration slowed at first and then accelerated to a level above the resting rate.

4. Gill deafferentation distinguished a short-latency reflex cardiac inhibition from a weaker long-latency slowing of central origin. The reflex, it is suggested, was mediated by branchial chemoreceptors. The existence of the central response was confirmed by occluding the conus with a clamp.

5. It is suggested that bradycardia in response to anoxia has a significance in relating cardiac output to the minute volume of water flow, thus ensuring adequate loading of the haemoglobin in the blood leaving the gills.

I am most indebted to the Nuffield foundation for a grant that has enabled equipment and technical assistance for this project to be purchased. In addition, thanks are due to the Medical Research Council of New Zealand who have supported this work. To Mr Vic Hansen I am perennially grateful for the supply of live dogfish. To my departmental colleagues, and in particular to Associate Professor J. R. Robinson, I am indebted for much helpful discussion.

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CENTRAL MECHANISM OF HEARING IN INSECTS

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INTRODUCTION

The electrical responses to sound stimuli have already been recorded from the auditory nerve bundle in several kinds of insect, in Orthoptera by Pumphrey & Rawdon-Smith (1936) and Haskell (1956, 1957), in Lepidoptera by Haskell & Belton (1956) and Roeder & Treat (1957), and in Hemiptera by Pringle (1953). The central mechanism of hearing, however, has not so far been much explored. Quite recently the present authors (Katsuki & Suga, 1958, 1960) studied electrophysiologically the problems of directional sense and frequency analysis in the tympanic organ of an insect by recording activities of the peripheral auditory neurons. The central mechanism has been further studied, and this paper is concerned with the experimental results. Three problems have particularly been posed: frequency analysis of sound, directional sense and central inhibition.

MATERIAL AND METHOD

The experiments were performed on *Gampsocleis buergeri* (Tettigoniidae), because of its large size and ready availability.

The insect was pinned on its back on a cork board and the ventral exoskeleton covering the nerve cord was removed. The tracheae distributing along the nerve cord were separated from the latter and the non-auditory inputs were also severed. The operated animal was placed about 50 cm from the loud-speakers and the sound was delivered from its left side in a sound-proofed room which was air-conditioned at about 26° C.

The impulses in response to sound stimuli were recorded from the connectives which were hooked up in the air with a 200 μ silver wire electrode mounted on a micromanipulator. In order to trace the auditory tract in the nerve cord, two different parts of the cord were simultaneously hooked up with two recording silver wire electrodes and the electrical response of each part was led through an amplifier to two beams of an oscilloscope. The sound wave and the time signal were indicated on a third beam simultaneously. The indifferent electrode was a silver wire placed on wet cotton on the abdominal segments from which the exoskeleton was removed.

Most records were photographed on a running film. By such a recording method, the difference in the response pattern and the time delay between the responses recorded from two different parts could be measured with some accuracy and the functional disposition of the auditory tract in the cord could be explored. In order to study the responses from the tympanic and cercal nerves separately, one or other of them was cut in most experiments.

The stimulating and the recording equipment used in the present work was the same as that described in the previous papers (Katsuki, Watanabe & Suga, 1959; Katsuki & Suga, 1960).

RESULTS

(1) *Response in the nerve cord*

When the recording was made from the thoracic connectives of *Gampsocleis buergeri*, spontaneous discharges of several units were always observed, their spike heights being various. The responses to tone bursts were seen only at the onset of sound among spontaneous discharges. Interfering impulses from regions other than the tympanic nerve were eliminated by cutting the rostral and caudal parts of the hooked-up connectives and the other peripheral nerves except the tympanic. Thus only the responses to tone bursts remained, the size of impulses ranging between 1 and 3 mV. In the tympanic nerve, the train of impulses lasted as long as the stimulus sound continued. In contrast, in the thoracic connectives the responses were evoked only at the onset of sound, that is the 'on'-type response. The conduction velocity measured at the suboesophageal–prothoracic connective was found to be about 6 m./sec. Pumphrey & Rawdon-Smith (1937) and Roeder (1948) have already reported that the conduction velocities of the cercal nerve and the giant fibre in a cockroach range from 2 to 3 m./sec. and 6 to 7 m./sec. respectively. Therefore, from the phasic discharge pattern, the large spike height, and the conduction velocity, it may be reasonable to conclude that the impulses originate from the large fibre in the connective.

The hair sensilla on the cerci can, as is already known, respond readily to low-frequency sound and the impulses evoked in the cercal nerve by sound are transmitted to the abdominal cord through the last (6th) abdominal ganglion. Our records, which were obtained from the abdominal nerve cord, always showed the distinct discharges of two units, the sizes of which ranged between 2 and 4 mV. The response pattern of the output of the last abdominal ganglion was more phasic as compared with that of the input. The conduction velocities of the fibres were about 6 m./sec. It was thus confirmed electrophysiologically that there were two auditory large fibres in the abdominal nerve cord.

(2) *Auditory large fibre*

The auditory large fibres in the nerve cord are divided into two: the auditory T large fibre and the auditory C large fibre.

(*a*) *T large fibre*

The patterns of the responses recorded from the connectives between the brain and the metathoracic ganglion were very similar. The impulses immediately evoked by the activity of the tympanic nerve at the prothoracic ganglion seemed to be conducted to the rostral and caudal ganglia through one and the same large fibre. In the hope of confirming this the impulses were recorded simultaneously from the connectives rostral and caudal to the prothoracic ganglion, that is to say, one recording electrode hooked up the suboesophageal–prothoracic connective and the other the ipsilateral meso–metathoracic connective. The descending impulses from the brain and the ascending impulses from the cercal nerve were excluded by cutting the rostral and

caudal parts of the connectives beyond the electrodes. The contralateral thoracic connectives and all the peripheral nerves except the ipsilateral tympanic were cut so as to leave only the simple system consisting of the unilateral tympanic nerve and the ipsilateral thoracic connective. The discharge pattern of the ascending impulses from the ganglion was exactly the same as that of the descending ones for any sound stimulus. The impulse sent to the suboesophageal ganglion is always delayed by 0·7 msec., compared with that directed to the mesothoracic ganglion. Thus it was confirmed that the impulses immediately evoked at the prothoracic ganglion were conducted rostrally and caudally on one and the same fibre.

Further attempts were made to discover whether an auditory large fibre of this type extends from the brain to the metathoracic ganglion. One of the electrodes hooked up the brain–suboesophageal connective and the other the meso–metathoracic connective. Fig. 1 represents an example of such simultaneous recordings. In each record of the figure, the upper and middle traces represent respectively the impulses sent up to the brain and down to the metathoracic ganglion, and the lower trace represents the sound stimulus. The frequency of the sound stimulus was changed as shown by the figures at the left side of each column. Good coincidence in the discharge patterns as between the upper and the middle traces is seen for the responses to all frequencies. The time delay between the impulses on the upper and middle traces was always 0·3 msec. and the spike heights were the same. This result shows that the auditory T large fibre runs in the cord as far as from the brain to the metathoracic ganglion and that the impulses in this fibre are initiated at the prothoracic ganglion, probably only by the activity of the tympanic nerve, and are conducted to the brain and to the metathoracic ganglion in exactly the same manner. Thus the information about the sound which stimulated the tympanic organ is sent up to the brain after about 12 msec. and down to the metathoracic ganglion after almost the same time from the arrival of the sound at the tympanic organ. The conduction velocity of this fibre was about 6 m./sec., measured at any part of the nerve cord.

The T large fibre does not extend to the abdominal ganglia, because no response originating from the tympanic organ was found beyond the metathoracic ganglion.

(*b*) *C large fibre*

The discharge pattern of the response evoked by impulses in the cercal nerve was found to be similar at all connectives from the last abdominal to the mesothoracic ganglion. The simultaneous records obtained from the connectives rostal and caudal to the metathoracic ganglion, however, did not show one-to-one correspondence of impulses. The responses recorded from the two connectives indicated that they consisted of the impulses of two large fibres. The number of impulses was always less at the thoracic connective than at the abdominal one, but the number of spontaneous discharges was just the reverse. From these results it may be said that these large fibres do not extend beyond the metathoracic ganglion, where they have synapses.

The impulses recorded from the metathoracic–abdominal connective, however, showed a perfect one-to-one correspondence with those from the V–VI abdominal connective in responses to sound and also in spontaneous discharges. The impulses at the rostral end of the abdominal cord were always delayed by 2·2 msec. relative to those at the caudal end. The conduction velocities of these fibres were almost the same,

about 5·8 m./sec. Thus the abdominal nerve cord has two pairs of similar auditory large fibres which run to the metathoracic ganglion from the last abdominal ganglion. Those auditory large fibres are called the primary auditory C large fibres in this paper.

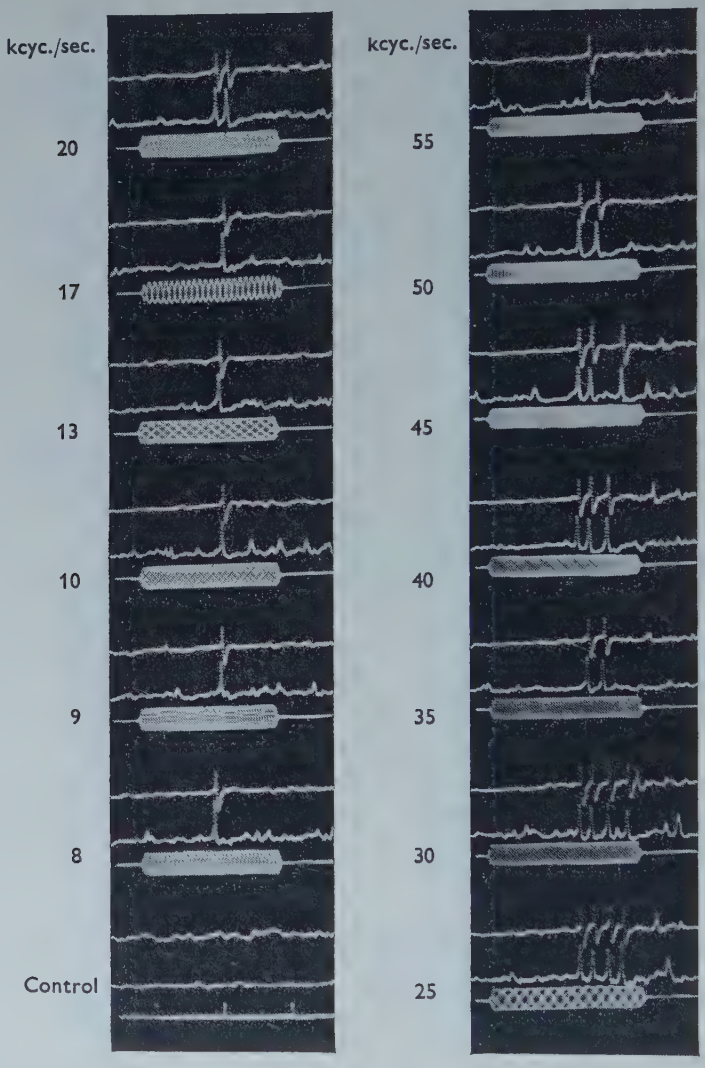

Fig. 1. Responses of the auditory T large fibre to sounds of various frequencies shown at the left side of each column. The upper and middle beams represent the responses recorded from the brain–suboesophageal connective, the ipsilateral meso–metathoracic connective respectively, and the lower beam represents the sound wave. Time signal (at the bottom of the left column), 10 msec.

A pair of auditory large fibres were, as described above, found in the meso–metathoracic connective. These fibres were activated at the metathoracic ganglion by the primary C large fibres. For this reason the auditory large fibres between the meso- and metathoracic ganglia are called the secondary auditory C large fibres. In the

nerve cord rostral to the mesothoracic ganglion, the C large fibre tract was not found electrophysiologically, but the activities evoked by low-frequency tone bursts were observed on several small fibres.

(3) *Interaction between impulses from the tympanic nerves of opposite sides*

The tympanic nerve in Orthoptera consists of about 100 nerve fibres (Vogel, 1921). When the tympanic nerve was hooked up with a silver wire electrode, the grouped discharges of those fibres were observed as long as the sound lasted. However, in each fibre a sigmoid relation was found between the sound intensity on a decibel scale and the number of impulses per sec. The information about the sound intensity can be thus signalled to the central nerve cord. On the other hand, the responses of the T large fibres were phasic, so it may be that the information about the intensity is not sent to the brain in the same form as it has in the tympanic nerve.

In order to study in detail the responses of the T large fibres, a pair of connectives between the suboesophageal and prothoracic ganglia was hooked up on the electrodes, the ascending impulses from the rear ganglia being eliminated by cutting the connectives. There remained both the tympanic nerves, the prothoracic ganglion, and both the ascending connectives. In Fig. 2 the upper and middle traces show the impulse discharges of the tympanic large fibres of the right and left sides respectively. The lower trace shows the wave form of the delivered sound, the frequency of which is 13 kcyc./sec. A and B represent respectively the responses before and after cutting the left tympanic nerve. The T large fibre of the left side, which was activated by the impulses sent up from the tympanic nerve nearer to the loud-speakers, sent more impulses than that of the right side (A). Here a very interesting phenomenon was found as a result of cutting the left tympanic nerve (B): no impulse discharge was found on the left side but in response to the same sound stimulus a remarkable increase in the number of impulses was found on the right side. When the right tympanic nerve was cut, the reverse effect was observed. When there were many impulses in a response to sound, the increase in number of impulses after cutting the nerve was not so marked, whereas when the number of impulses in the response was fewer, the increase after the cut was more marked. It was found that the impulses delayed by more than 4·6 msec. after the first impulse in the response were suppressed by the impulses of the contralateral tympanic nerve. This phenomenon shows that impulses of the tympanic nerve on one side have an inhibitory effect on the contralateral T large fibre.

In three cases out of twenty-six the impulses in the T large fibre remained even after cutting the ipsilateral tympanic nerve, though the number was small. Disappearance of these remaining impulses after cutting the contralateral tympanic nerve proved that they were evoked by the activity of the contralateral nerve. They were delayed by several milliseconds as compared with the ipsilaterally evoked impulses. The shortest delay was 3·3 msec. When the contralaterally evoked impulse was observed, an attempt was made to discover whether the contralaterally and ipsilaterally evoked impulses converged on the same T large fibre. No difference in the spike height and no summation of spikes were found among them. The delay of the first spike transmitted from the contralateral tympanic nerve was always about 10 msec. from the onset of the sound stimulus, but the spike with this delay was not found

regularly in the response of the T large fibre before cutting the ipsilateral tympanic nerve. These facts suggest that the ipsilaterally and the contralaterally conducted impulses in the tympanic nerves activate one and the same T large fibre. However, there still remains the question of whether the inhibitory effect is exerted upon the tympanic nerve. By recording the electrical activity from the latter it was confirmed that the inhibitory effect of the tympanic nerve on one side does not extend to the tympanic nerve of the opposite side.

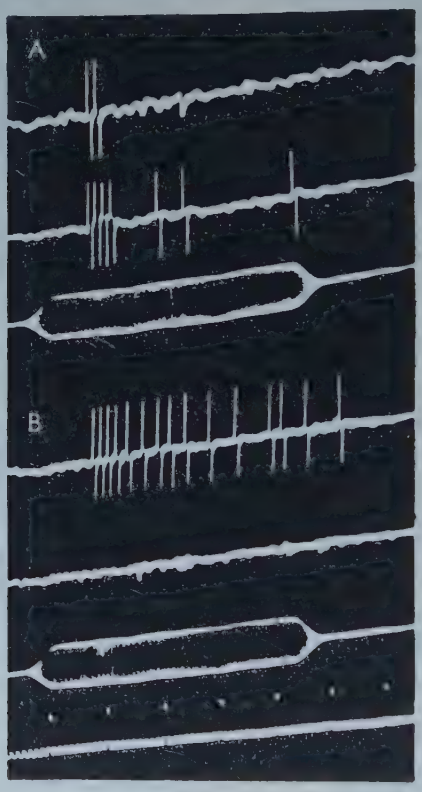

Fig. 2. Inhibitory effect of the tympanic nerve on the contralateral T large fibre. In A and B the upper and middle beams represent impulse discharges of the right and left large fibres respectively. A is before and B is after the elimination of the inhibitory effect by cutting the left tympanic nerve. The sound stimulus on the lower beam is 13 kcyc./sec. in both A and B. Time signal, 10 msec. See text.

Therefore it is highly probable that the T large fibre receives not only the excitatory effect from the ipsilateral tympanic nerve at the prothoracic ganglion but also the inhibitory and weak excitatory effects from the contralateral nerve. The irregularity of the contralaterally evoked impulses tells us that the inhibitory effect may be varied by intrinsic factors.

The discharge pattern of the T large fibre was phasic as described above, but it was due to the inhibitory effect of fibres from the opposite side. When the contralateral tympanic nerve was cut, though the T large fibre responded with increased impulses, the response adapted so that the train of impulses lasted at most only for the

first 0·1 or 0·2 sec. of continuous tones of 0 db. on our scale (corresponding to about 100 db. above average human threshold for 1 kcyc./sec.), the frequencies being higher or lower than the characteristic frequency of the tympanic organ (Katsuki & Suga, 1960); the discharge continued for about 1 sec. in the response to sound of the characteristic frequency at the same intensity.

On the other hand, such an inhibitory effect was not disclosed in the primary C large fibres. When one of the cercal nerves was cut, only a few impulses in response to sound remained in the ipsilateral large fibres and many others disappeared. On the contralateral large fibres, a decrease of a few impulses was observed as compared with the original response. Therefore the interaction between the impulses from the two cercal nerves was only excitatory.

(4) *Response ranges of the auditory large fibres*

As already reported (Katsuki & Suga, 1958, 1960), the tympanic organ of *Gampsocleis buergeri* responds to the sounds of 0·6–75 kcyc./sec. and among them most sensitively to 10 kcyc./sec. sound. The thresholds of the T large fibre for sounds of various frequencies were measured at the brain–suboesophageal connective after cutting off the brain. The frequency range responded to was found to be from 2 to 60 or 70 kcyc./sec. and the most effective frequency from 10 to 20 kcyc./sec. In the previous paper (1960), the authors reported two ranges of response in the tympanic nerve of this insect. The response range of the T large fibre was found to be almost the same as one of them, i.e. that in response to higher-frequency sounds. The threshold of this fibre for the sound of the most effective frequency was not higher than, but almost the same as, those of the tympanic neurons. The responses which might be evoked by the impulses of neurons of the other type in the response range were not found in the central nerve cord, at least not in the large fibre.

On the other hand, the threshold curve of the cercal hair sensilla covers the range of sounds from below 30 up to 2000 cyc./sec. Sounds from 400 to 800 cyc./sec. were most effective to this sense organ. The frequency range of the primary C large fibre was the same as that of the peripheral cercal nerve. No difference was found in the threshold curve between the cercal nerve and the primary C large fibre.

Coming now to directional sense, it was to ultrasonic waves that the largest difference in sensitivity between a pair of tympanic organs of the locust* was found (Katsuki & Suga, 1960). Therefore the difference in the response range between the two T large fibres of *Gampsocleis buergeri* can be anticipated.

Simultaneous records were made from a pair of the suboesophageal–prothoracic connectives. The threshold of the left T large fibre (●—●), directed towards the sound source, was lower for all frequencies as compared with that of the opposite one (○—○), as shown in Fig. 3. After cutting the left tympanic nerve the response range of the right T large fibre (○– – –○) did not change in spite of the marked increase in the number of impulses. This fact shows that some of the tympanic nerve fibres can operate to modify the content of the information sent by the contralateral T large fibre without changing its response range, because the inhibitory effect from the contralateral tympanic nerve suffers some time delay.

* *Locusta migratoria danica* in the previous paper was erroneous. It should be *L. migratoria manilensis*.

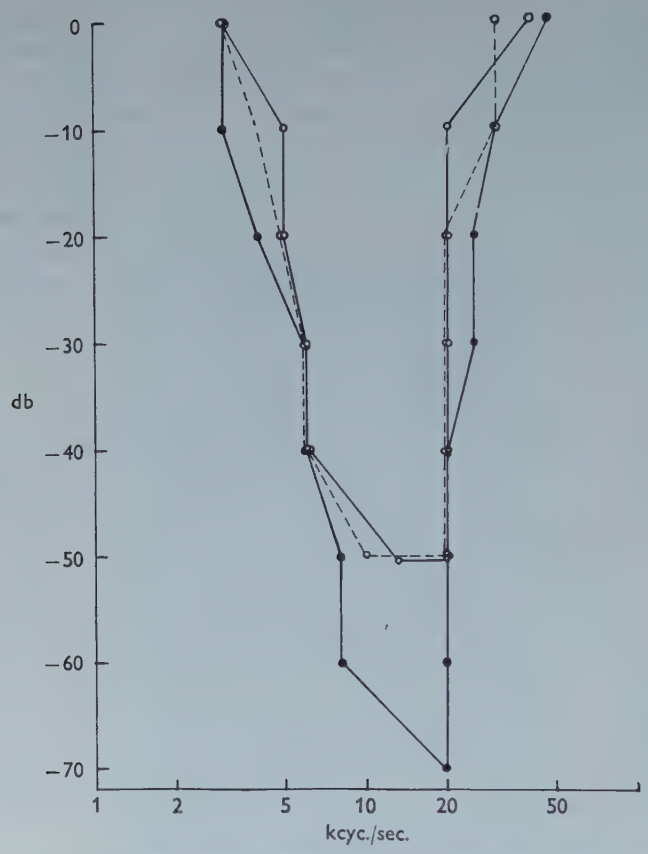

Fig. 3. Difference in response ranges between the left (●—●) and right (○—○) T large fibres. The response range of the right T large fibre does not change in spite of the remarkable increase of impulses evoked by cutting the left tympanic nerve near to a sound source (○– – –○). The ordinate and abscissa represent respectively the intensity and frequency of the sound.

(5) *Modification of information in the T large fibres*

The extent of modification of information in a pair of the T large fibres by the inhibitory interaction already described was next studied. The tone bursts used had a constant duration of 70 msec. and either their intensity or their frequency was varied. In A of Fig. 4 the ordinate and the abscissa represent respectively the number of impulses in the response and the frequency of the sound in kcyc./sec. The intensity of the sound was kept constant at 0 db. A difference in the number of impulses was found between the responses of both left (○– – –○) and right (○—○) fibres only to sounds of from 10 to 20 kcyc./sec., and the most marked difference at 17 kcyc./sec. When the left tympanic nerve (nearer to the loud-speakers) was cut, the response of the left fibre (responding with more impulses before cutting) disappeared completely, and on the opposite right (●—●) fibre a marked increase in the number of impulses immediately appeared as a result of the release of the inhibitory effect from the left tympanic nerve. The frequency range of sounds which activated the T large fibre was from 2 to 65 kcyc./sec. The increase of impulse discharge after the elimination of the inhibitory effect was found in the response to sounds of from 5 to 30 kcyc./sec., but

the marked difference in the number of impulses between the two large fibres under inhibitory effect from the opposite side was found in the narrower range as described above. This range almost coincides with the frequency range most effective to the tympanic organ and also with the dominant frequencies involved in the stridulatory sound of the group.

In B of Fig. 4 the ordinate and abscissa show respectively the number of impulses in the response and the intensity of the sound in decibels. As the change in the number of impulses was small in the frequency regions both lower and higher than the most effective region, the curves were plotted for the responses to the most effective frequency, at which the change was very marked. The right T large fibre (○—○), situated farther from the sound source, responded with only one spike from 0 to −50 db., while the left one (○- - -○) responded with several spikes even to a weak sound. When the left tympanic nerve was cut, the right T fibre showed many impulses, (the response evoked by the right tympanic nerve alone) but no change of the threshold (●—●).

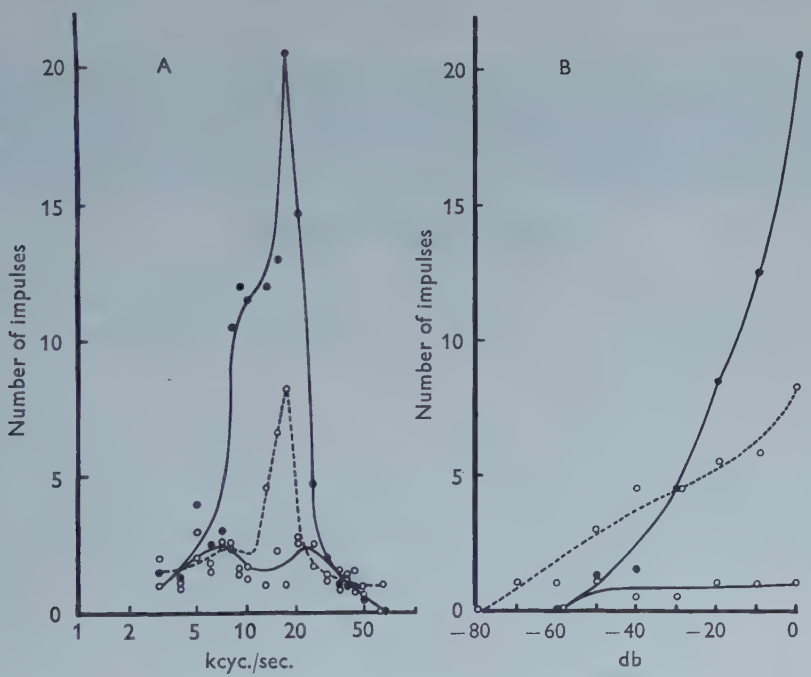

Fig. 4. Change in the number of impulses (ordinate) in the T large fibres before (○—○) and after (●—●) cutting the left tympanic nerve. The number of impulses in the left large fibre is shown on a curve with open circles and a dotted line. In A, the intensity of sound is kept constant and the frequency is changed (abscissa). In B, the frequency is kept constant at 17 kcyc./sec. and the intensity is changed (abscissa). See text.

(6) *Response to the stridulation of the group*

The responses of the tympanic nerve to the stridulation of the group show, as already known (Haskell, 1956, 1957; Katsuki & Suga, 1958, 1960), good synchronization with the pulsatory sounds which compose the stridulation. What information

about the stridulation is sent through the nerve cord to the brain, however, is still obscure.

The responses were simultaneously recorded from the T large fibre and also from the primary C. Several stridulating males (*Gampsocleis buergeri*) were placed in a bamboo cage at a distance of about 50 cm. from the insect being recorded. The T large fibre discharged synchronously with the pulsatory sounds, but the primary C did not. From this fact it can be said that auditory communication in the group is mainly mediated by the tympanic organ.

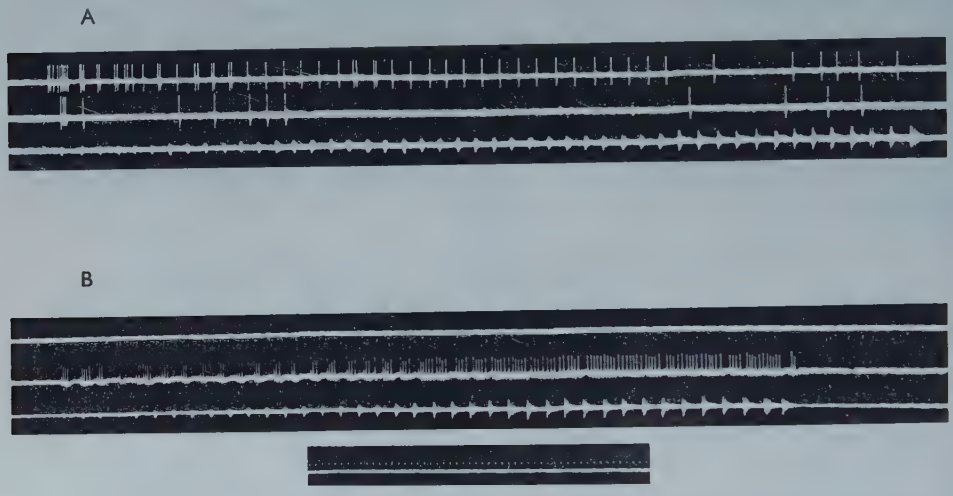

Fig. 5. Responses to the stridulation of the group. The upper and middle beams represent the impulse discharges of the right and left T large fibres respectively and the lower beam represents the stridulation of a male placed at the right side of the recorded female. A and B show the responses of the T large fibres before and after cutting the right tympanic nerve. Time signal, 10 msec. See text.

When the responses to stridulatory sounds were simultaneously recorded from a pair of the T large fibres, the impulses of the T large fibre closer to the source showed a distinct one-to-one correspondence with the pulsatory sound, but those of the opposite fibre did not (A of Fig. 5). When the source was moved from the side to a position in front of the insect being recorded, the impulse discharges of both connectives became almost the same. After one of the tympanic nerves was cut, the contralateral T large fibre responded to the pulsatory sound with a volley of impulses. Only three or four impulses were synchronized with the initial weaker pulsatory sounds while a train of impulses was observed to the later stronger ones (B of Fig. 5).

DISCUSSION

As described above, the impulses arising in the tympanic nerve in response to sound evoke spikes in the T large fibre in the cord. However, the records obtained from the suboesophageal–prothoracic connective showed that those impulses were transmitted not only to the large fibre, but also to a small fibre, the size of whose impulses, whenever found, was less than half of that of the large fibre. The response range of the small fibre was nevertheless the same as that of the large one.

On the other hand, besides two C large fibres described above, one or two units were found in the abdominal nerve cord. In this paper, however, the description is limited only to the auditory T and C large fibres which were observed very clearly and constantly.

(1) *Auditory large fibres*

The functional analysis of the nerve cord in Arthropoda has been thoroughly carried out in the crayfish by many authors (Wiersma, Ripley & Christensen, 1955; Wiersma, 1958; Furshpan & Potter, 1959*a*, *b*; Kennedy & Preston, 1960; Preston & Kennedy, 1960; Hughes & Wiersma, 1960). A T-shaped interneuron was found anatomically in the abdominal nerve cord by Allen (1894). Hughes & Wiersma (1960) reported that certain abdominal peripheral nerves evoked simultaneously ascending and descending impulses in one and the same abdominal nerve fibre. The anatomical study of the nerve cord of *Gampsocleis buergeri* by the present authors has revealed that there are T-shaped neurons and a pair of large fibres (about 27 μ in diameter) which lie in the cord between the brain and the metathoracic ganglion. The suboesophageal–prothoracic connective has 8 or 9 fibres larger than 25 μ in diameter and the largest of these is 38 μ. In the abdominal nerve cord five large fibres are found with a diameter of 20–27 μ. But it is difficult at present to determine which of these is the auditory large fibre.

(2) *Directional sense*

The inhibitory interaction between the tympanic nerves of opposite sides has no effect on the difference between the thresholds of left and right T large fibres, in spite of the large modification of information carried by those fibres. From the point of view of the threshold the difference in responses between the large fibres therefore follows that between the two tympanic nerves. If the tympanic large fibres were released from the mutual inhibitory interaction, each of them could send a train of impulses and the information about the intensity difference would simply correspond to that in the tympanic nerves. In fact, one tympanic nerve firing with many impulses will activate strongly the ipsilateral T large fibre while strongly suppressing the activity of the contralateral one. By such a mechanism, the information about a sound source is increased and the information is sent quickly to the brain through a pair of T large fibres. Such an inhibitory interaction may be one of the most important factors in the mechanism of directional sense. The inhibitory interaction was also clearly observed in the nerve cord of *Homoeocoryphus lineosus*.

(3) *Frequency analysis*

It is difficult to understand that about 100 tympanic neurons connect with only two of the central neurons. However, during the course of the present experiments there was no sign that some of the tympanic nerve fibres or any central small fibres (except for two fibres described above) sent impulses to the brain beyond the prothoracic ganglion. The response ranges of the T large fibres were narrower than those of the tympanic nerve (quite recently, the same phenomenon in the locust and cricket was noticed by Horridge (1960)) and coincided with one type of response range of single tympanic neurons. The characteristic frequency was always found to be between 10 and 20 kcyc./sec. There was also no evidence that the central neurons activated by

the tympanic nerve sent impulses into the brain with different response patterns for different frequencies of sounds. Neurons having different characteristic frequencies were not found in the peripheral nerve of a locust, notwithstanding that the sensilla of the tympanic organ are divided into three groups (Suga, 1960). However, from his observation that the characteristic frequency of an ascending central neuron is shifted by a continuous pure tone, Horridge (1960) has suggested the existence of two groups of sensory neurons in the tympanic organ or in the ganglion, that is to say, the existence of a mechanism of frequency analysis. In *Homoeogryllus japonicus*, the present authors measured the thresholds for tone bursts with various frequencies before and after a continuous pure tone of a certain frequency was delivered and found that the thresholds became relatively high for tone bursts with a background tone. The authors think that Horridge's suggestion is quite reasonable that there are at least two groups of sensory neurons, but the fact that impulses from two groups are transmitted in only one central neuron does not confirm the possibility of frequency analysis even if the response range of the neuron was shifted by a continuous tone. If each of the central neurons which are activated by the tympanic neurons has a different frequency range and characteristic frequency, we can entertain the possibility of frequency analysis in the tympanic organ and its neural pathway. As things stand now, we shall have to look over the whole body surface of an insect if we believe in the possibility of frequency analysis in insects.

The large fibre activated by sound reception of the cercal hair sensilla sends up the information to the mesothoracic ganglion through the relay at the metathoracic ganglion. The information sent up to the mesothoracic ganglion is transmitted to some fine fibres in the ganglion and finally sent into the brain. The response ranges of these C large fibres correspond with the threshold curve of the whole cercal nerve.

The difference in the frequency range between the two sound receptive organs, the tympanic organ and the cercal hair sensilla is very remarkable, and they send impulses into different auditory large fibres. Therefore it will be concluded that the insect can analyse sound frequencies to some extent, probably by the spatial and temporal pattern of impulses. The tympanic organ together with its neural network seems to be elaborated to receive the stridulatory sound of the group.

In order to support the interpretation of the central interaction described above, several pharmacological agents which have a specific action on inhibitory or excitatory synapses were applied to the prothoracic ganglion and the change in impulse discharges of the auditory T large fibres was examined. The results afforded evidence that the central interactions described above were synaptic events. The details of the pharmacological experiment will be reported in a separate paper.

SUMMARY

1. The impulses from the tympanic organ are transmitted at the prothoracic ganglion to a central neuron, the auditory T large fibre, which lies in the cord between the brain and the metathoracic ganglion. The impulses in the T large fibre are conducted rostrally and caudally with the same discharge pattern. Information is sent up to the brain, and down to the metathoracic ganglion, after a delay of about 12 msec.

2. The impulses from the cercal hair sensilla are transmitted to two similar auditory C large fibres which lie in the cord between the metathoracic and last (6th) abdominal ganglia and are then sent up to the mesothoracic ganglia by other auditory large fibres.

3. Central inhibitory interaction between the impulses from the tympanic nerves of the two sides are shown by a marked increase of impulses in the T large fibre following section of one of the tympanic nerves. No inhibitory interaction is found between the impulses from the two cercal nerves.

4. The auditory T large fibre receives not only the excitatory effect from the ipsilateral tympanic nerve at the prothoracic ganglion, but also the inhibitory and weak excitatory effects from the contralateral one.

5. The response range of the T large fibre is narrower than the threshold curve of the tympanic nerve and corresponds with one type of response range in the tympanic neurons. The response ranges of the C large fibres correspond closely with the threshold curve of the cercal nerve.

6. A large difference in threshold between the two T large fibres is found in the response to sound incident from the side. The number of impulses in the T large fibre nearer to the sound source is greater than in that farther from the source.

7. The difference in the number of impulses between the two T large fibres is most marked in the response to sound of the frequency which is dominant in stridulation. This difference is due to the mutual inhibitory interaction of neurons which modifies the number of impulses without changing the threshold of the tympanic large fibre.

8. It is suggested that the central inhibitory interaction increases the information about a sound source and plays an important role in the mechanism of the directional sense.

9. The stridulation of the group activates the tympanic nerve and evokes synchronized discharge in the T large fibre, but scarcely at all in the primary C large fibre. The tympanic organ and its neural network seem well adapted to reception of stridulation.

10. It is concluded that though neither of the two sound receptive organs—the tympanic organ and the cercal hair sensilla—can perform frequency analysis, the insect may be able to do so by making use of both organs, since they have different frequency ranges and are served by different auditory large-fibre tracts.

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COLONIAL RESPONSES OF HYDROID POLYPS

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INTRODUCTION

There are many reports in the literature confirming the existence of colonial co-ordination in the Coelenterata. Studies have been made on co-ordinated polyp responses and on the spread of luminescent waves across a colony, activities presumably mediated through nerve nets. Several authors have described contraction of polyps other than the ones directly stimulated in colonial Hexacorallia (Klunzinger, 1877; Duerden, 1902; Matthai, 1918; Abe, 1939; Hyman, 1940; Horridge, 1957); and propagated waves of polyp retraction have been described for the Octocorallia (Milne-Edwards, 1835; Krukenberg, 1887; Parker, 1920*b*; Hiro, 1937; Gohar, 1940; Horridge, 1956*a*, *b*, 1957; Broch & Horridge, 1957). Most of the reports merely mention the occurrence of colonial responses, however, and only Krukenberg, Parker, and especially Horridge have analysed these reactions in any detail.

The luminescent waves which traverse colonies of certain alcyonarians have been the most studied colonial responses of coelenterates, probably owing to the ease with which they can be observed and, with modern methods, accurately measured. Among the works dealing with these luminescent responses are those of Panceri (1872), Harvey (1917), Parker (1919, 1920*a*, *b*), Moore (1926), Honjo (1944), Buck (1953), Nicol (1955*a*, *b*, 1958) and Davenport & Nicol (1956).

Among the Anthozoa, only for the alcyonarian *Veretillum* does there appear to be histological evidence for a colonial nervous system (Niedermeyer, 1914). Kassianow (1908) failed to find nervous connexions between polyps in *Alcyonium*, but the recent paper by Horridge (1956*a*) on this genus gives physiological evidence that such connexions exist. Hyman (1940), discussing the Madreporaria, states that, on the basis of behavioural responses, 'the presence of a nerve net throughout the colony must be assumed'. It appears probable that the dearth of histological evidence for colonial nervous systems in Anthozoa is only a result of the scarcity of histological studies devoted to colonial forms.

The other coelenterate class with colonial representatives is the Hydrozoa. Among the Hydrozoa the siphonophores offer the most highly specialized colonies in the Animal Kingdom. There now appears to be good reason to regard *Velella* and its relatives (the Chondrophorae of Hyman (1940)) as single modified polyps and not colonies in the usual sense of the word (Garstang, 1946; Mackie, 1959), and these forms will not be considered here. Nervous connections between at least some of the members of a colony have been histologically detected in the remaining siphonophores (Schaeppi, 1898; Mackie, 1960*a*, *b*), and responses of many individuals following stimulation at one point has been described by Bigelow (1891), Schaeppi (1898), Schneider (1902), and Mackie (1960*a*, *b*).

There appear to be few reports on colonial co-ordination in hydroids. Föyn (1927) described contraction of all members of *Clava* colonies when the stolon is mechanically stimulated, and he used such responses to delimit the bounds of one colony when several colonies grew from a common intertwined mat of stolons. Co-ordinated responses of polyps, especially the spiral zooids, have been reported by Wright (1856) and Schijfsma (1935), among others, for *Hydractinia*. Zoja (1891), in a commonly overlooked paper, described propagated polyp withdrawal following electrical stimulation in *Corydendrium*, *Coryne*, *Eudendrium*, *Tubularia*, and *Campanularia*, and he studied this reaction in some detail in *Pennaria* and *Podocoryne*. His results are discussed in the parts of this paper dealing with some of these same genera. Evidence for the presence of a neural substrate which could mediate these responses is given by the report of ganglion cells in the coenosarc of *Syncoryne* (Citron, 1902) and *Cordylophora* (Mackie, 1961). Jickeli (1883*a*) reported ganglion cells in *Eudendrium* in the 'hydrophyton' (a term designating both the hydrocaulus and the hydrorhiza according to Allman (1871)). Jickeli, however, described the hydrophyton as being capable of movement and shape changes, so he may have been referring to only that portion of the hydroid immediately below the hydranth proper.

The following study was begun in an attempt to augment our limited knowledge of colonial responses in coelenterates.

MATERIALS AND METHODS

The animals used in this study were the colonial hydroids *Pennaria*, *Syncoryne*, *Tubularia*, *Cordylophora*, *Podocoryne*, *Hydractinia aggregata* and *H. echinata*. Species were identified with the aid of Fraser's monographs on American hydroids (1937, 1944). All experiments were performed on colonies submerged in sea water or, in the case of *Cordylophora*, in dilute sea water. Mechanical stimuli were given to the colonies by pinching with forceps or by prodding with a fine glass rod. Electrical stimuli were delivered to the colonies by fine silver wires (0·2 mm. in diameter) insulated to the tip with lacquer. When stimulating colonies of *Hydractinia* or *Podocoryne* which form two-dimensional sheets, the electrodes were placed about 1 mm. apart on the stoloniferous mat of the colony. For the other species, the electrodes were placed one on each side of the slender stolons or stalks of the colony. The electric stimuli in the experiments on *H. echinata* were produced by a condenser-discharge apparatus which gave pulses with a half-amplitude duration of 0·05 msec. For all other species the electric stimuli were square pulses produced by a Grass S4 stimulator and were, unless otherwise specified, 0·5 msec. in duration.

Observations of polyp responses were made visually. Techniques used uniquely on one species will be described in the section devoted to that species.

RESULTS

I. *A species with both a through-conducting and a local polyp co-ordinating system*

Gastropod shells carried by hermit crabs, collected at Woods Hole, are often encrusted with colonies of *Hydractinia echinata* Fleming. Such colonies can contain several thousand nutritive polyps or gastrozooids, each about 1 mm. high. Scattered

among the gastrozooids are numerous reproductive polyps, the gonozooids. Spiral zooids, presumably defensive polyps, are frequently found around the aperture of the gastropod shell.

(*a*) *Mechanical stimulation*

Prodding a gastrozooid with a glass rod may give no polyp responses, or a contraction of one or a few tentacles, or contraction of both the tentacles and the hydranth. This contraction often spreads to neighbouring polyps, and several hundred polyps can contract following such stimulation. Lightly pushing on the stoloniferous mat causes a similar local wave of polyp contraction. The polyp contraction often spreads in patches, with contraction in one group of polyps following contraction in another group after a brief but noticeable delay. Polyps included in a contracted area take about 30 sec. to relax. The conduction velocity of the waves of local contraction was not accurately measured, but it appeared to be about 1 cm./sec. If the area of contraction includes some spiral zooids, these become more tightly coiled and move closer to the stoloniferous base.

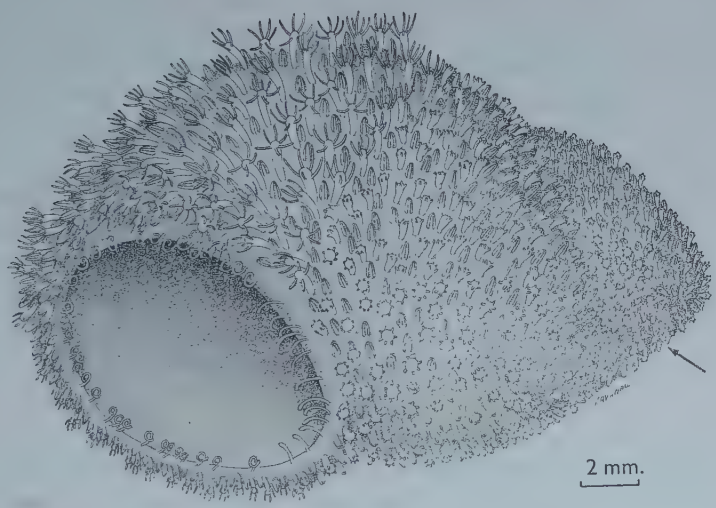

Fig. 1. A *Hydractinia echinata* colony as it would appear immediately following stimulation at the point marked by the arrow. A wave of polyp contraction which will affect all the polyps is shown as it is sweeping across the colony. The dactylozooids in the responding area are lashing.

When a single polyp is crushed with forceps or the stolon mat is damaged, a wave of polyp contraction is initiated which affects all members of the colony. When this wave reaches a spiral zooid, the spiral zooid does not coil more tightly but suddenly uncoils, lashing out, sometimes repeatedly (Fig. 1). The conduction velocity of such excitation appears considerably faster than that associated with local responses; spiral zooids near the stimulated area and those some distance away across the shell aperture from the stimulated area lash almost simultaneously. The different conduction velocities indicate activity in different conducting systems. The polyps relax slowly following injurious stimulation and often take several minutes to re-extend. Polyps far from the injured area frequently relax before those near the point of stimulation.

(*b*) *Electrical stimulation*

Local polyp contraction similar to that seen with mild mechanical stimulation can be evoked with an electric shock. Such contraction often spreads in patches, and spiral zooids included in a responding area coil more tightly. Although this response can be produced by a single shock, there is some summation of sub-threshold stimuli and several shocks, each 20–30% below threshold, can give a local contraction. A second shock following an effective one sometimes increases the size of the locally responding area. More often, however, it either has no effect or it causes contraction of the whole colony and lashing of the spiral zooids. Increasing the stimulus intensity much above threshold usually causes such colony-wide contraction.

On many occasions a local contraction of polyps cannot be obtained; even just-threshold stimuli evoke contraction of the whole colony and lashing of the spiral zooids. This response is always seen with stimuli of much above threshold intensity. At low frequencies of stimulation the spiral zooids lash once for each of the first few shocks. At frequencies above 2/sec., however, individual spiral zooids do not follow each stimulus, and the lashing of the spiral zooids of the colony becomes asynchronous, some following every other shock, some following every third. Usually only the first few stimuli of a series are effective. In a typical case, using a 1/sec. train of stimuli, the gastrozooids contracted to the first shock and the spiral zooids lashed for each of the first four shocks. The spiral zooids then ceased responding and the remaining polyps began to relax, even though the stimuli continued.

On two occasions, following strong, repetitive shocks, relaxation was continually interrupted by spontaneous recontraction of patches of polyps. The areas spontaneously contracting became smaller and the intervals between contractions lengthened with time until the colony was again quiescent. Complete relaxation took about 15 min. Such polyp behaviour appears analogous to the spontaneous waves of luminescence seen in sea pens following repeated stimulation (Buck, 1953; Nicol, 1955*b*, 1958; Davenport & Nicol, 1956).

II. *A species with a through-conducting polyp co-ordinating system*

Podocoryne carnea Sars resembles *Hydractinia echinata* in appearance, and similarly has spiral zooids around the aperture of the gastropod shell upon which it grows. Only a few experiments were done on this species. The colonies used were sent to Los Angeles from Woods Hole, and, although they did survive for several weeks in the laboratory, evaluation of these experiments must be made with the reservation that the colonies may not have been in fully prime condition.

The only response to either mechanical or electrical stimuli seen in these colonies was rapidly propagated, colony-wide contraction of polyps and lashing of the spiral zooids. The contraction of the gastrozooids was sometimes incomplete, occasionally no more than a brief jerk. When the polyps did contract, relaxation was often slower near the point of stimulation. The colony-wide contraction was quite all-or-none and strength–duration curves could easily be obtained for electric stimuli. The chronaxie from such curves was between 0·5 and 1 msec.

Zoja (1891) described polyp contraction in *Podocoryne carnea* following electrical stimulation as being simultaneous everywhere in the colony and occurring in two or

three separate contractions. In my experiments the conduction velocity of this species was rapid but not 'instantaneous', and no evidence of repetitive activity following electrical stimuli was seen.

III. *Species with local polyp co-ordinating systems*

(1) Cordylophora lacustris *Allman*

These hydroids were grown in sea water diluted to 10% by aged water from a fresh-water aquarium. Some of the original specimens were given me by Dr O. Kinne and others were personally collected from the Sacramento River near Antioch, California. They were kept at 20°–22° C. and fed several times a week with newly hatched brine shrimp. Under such conditions this species forms colonies consisting of a single stolon and, every 2–4 mm., short, upright stalks, each bearing one hydranth. The older polyps and coenosarc regress as the colony forms new polyps; a complete colony under these conditions usually contains three to six hydranths.

(*a*) *Mechanical stimulation*

If a polyp is probed with a glass rod, the polyp bends toward the rod, or one or more tentacles retract, or all the tentacles become depressed and the polyp shortened. The latter response will be called polyp contraction. Such activities do not affect neighbouring polyps. If a polyp is pinched with forceps or torn with a glass rod, a propagated wave of polyp contraction is seen which involves all members of these small colonies. In contrast to the high intensity of mechanical stimulation to a *polyp* needed to cause responses of neighbours, gentle prodding of the *stolon* of a colony causes an immediate wave of contraction, again affecting all members of the colony.

(*b*) *Single electric stimuli*

When a portion of the stolon is stimulated with a single shock of sufficient intensity, a wave of polyp contraction is initiated which propagates with an average velocity of 2·6 cm./sec. (22° C.). This response is not all-or-none (Fig. 2); both the number of polyps responding and the degree of the contraction in any one polyp increases with increasing stimulus intensity. The range over which increasing the stimulus intensity gives a greater response is small. In over half of twenty trials of an experiment involving several different colonies, increasing the stimulus intensity 25% above threshold gave complete contraction of all the polyps of the colony and in no instance was it necessary to double the stimulus intensity to achieve this end.

In most cases the effect of increasing the stimulus intensity is not smoothly graded. A just-threshold stimulus often causes approximately equal contraction in several polyps near the electrodes and frequently no one polyp can be made to respond individually. Slowly increasing the stimulus intensity in such a case gives no greater response until some critical level is reached, at which time more polyps respond and those of the initially responding group show greater contraction. In *Cordylophora*, therefore, groups of two to three polyps often form behavioural units with respect to stimuli applied to the stolon.

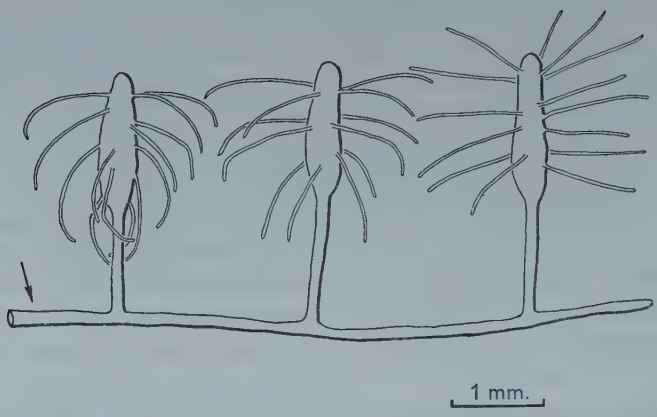

Fig. 2. A *Cordylophora* colony following a shock of not much above threshold intensity applied to the stolon at the point marked by the arrow. The polyp nearest the point of stimulation is almost completely contracted, the next polyp is less contracted, and the most distal polyp is unaffected by the stimulation.

(*c*) *Repetitive electric stimuli*

If two sub-threshold shocks are given to the stolon within 10 sec. of each other, one or more polyps will often respond. The intensity range over which this apparent summation of sub-threshold stimuli occurs is small, usually both stimuli have to be within 10% of the threshold intensity. The effect of a second shock following the first within 10 sec. is quite pronounced when using stimuli of intensity between threshold and that which gives maximum contraction of all the members of the colony. The second stimulus usually causes more polyps to respond and the already active polyps to contract more fully. If a second shock is given following a shock which evoked complete contraction of all members of the colony, it often delays relaxation of the polyps, although sometimes it has no apparent effect. Occasionally with such stimuli polyps near the electrodes show retarded relaxation while those farther away relax in a normal manner. It appears in such cases as though conduction of the second stimulus has been blocked somewhere in the colony.

Some of the activities of the polyps are apparently unaffected by the induced tentacle depression response. Feeding and defecation continue while a polyp is being stimulated even though they may be temporarily interrupted during the tentacle depression period. In one case, the middle of three polyps being observed captured a small crustacean. When stimuli of above threshold strength were applied to one end of the colony, all but the centre polyp showed a maximal response, the centre polyp not responding at all. This was the only observed instance where the excitation seemed to skip one polyp and continue to a more distal polyp. Usually feeding did not completely inhibit tentacle depression, but did lead to a lesser degree of contraction as compared to non-feeding polyps.

(*d*) *Changes in sensitivity*

If a colony is allowed a 1 min. rest between each stimulus or group of stimuli, the intensity required to give maximal contraction of all the members of the colony slowly increases during the experimentation and, at the end of a 2 hr. period, might

be twice what it was at the beginning. Small changes in the position of the electrodes do not affect this decreased sensitivity. A change in the sensitivity to electric stimuli is not seen if 5 min. are allowed between each stimulation.

If a number of supra-threshold shocks are given to the colony at short intervals, the polyps soon cease to respond. In one experiment, for example, the hydranths contracted following each of the first five stimuli delivered to the stolon at 2 sec. intervals and then began to relax, even though the stimulation continued.

(2) Pennaria tiarella (*Ayres*)

Pennaria colonies were collected from floats and pilings near the Hawaii Marine Laboratory. These colonies consist of a main stem which is several centimetres long and, on alternating sides of this stem, branches bearing polyps at 2 mm. intervals. Near the base of the colony the branches are longer and may have as many as eight hydranths, while distally they become shorter and the most distal branches have but a single hydranth. A complete colony has about fifty hydranths. The hydranths are 1 mm. high, and each has a distal row of seven to thirteen long filamentous tentacles and numerous short capitate tentacles scattered over the manubrium. Only freshly collected colonies were used in this study, as even in running sea water the colonies soon degenerated in the laboratory.

(*a*) *Mechanical stimuli*

Pennaria shows much spontaneous activity. Polyps often bend from side to side and individual tentacles are frequently elevated. If a polyp is lightly touched with a fine glass rod, the hydranth bends toward the rod or one or more of the filamentous tentacles rises up over the stimulated area. More forceful prodding causes a co-ordinated elevation of the row of long tentacles. Strong mechanical stimulation, such as pinching with forceps, evokes contraction of the polyp body and folding of all the tentacles about the hydranth. This response will be called polyp contraction. Strong mechanical stimulation of a polyp often leads to contraction of neighbouring polyps, and frequently a wave of polyp contraction affecting all members of the colony is thus initiated. Pinching the stem or one of the branches usually causes a wave of polyp contraction which spreads to all parts of the colony.

(*b*) *Single electric stimuli*

A single shock to either the stem or a branch can cause contraction of a few to many polyps which is by no means all-or-none (Fig. 3). The hydranth responses range from slow, partial elevation of the proximal tentacles, with relaxation in 10 sec. or less, to rapid, complete closure of the tentacles with relaxation taking from 40 to 180 sec. The magnitude of the polyp response is graded with distance from the stimulating electrodes; those polyps near the electrodes contract more than those near the periphery of the responding area.

The sensitivity of the colony usually declines greatly during a series of stimulations. The threshold was often found to have more than doubled between successive determinations, even though 1 min. had been allowed between each stimulus. This decreased sensitivity was not changed by small movements of the stimulating electrodes. After a long period of stimulation, leaving the colony quiescent for periods up to 1 hr. did

little to reduce the heightened threshold. Experiments with two pairs of electrodes on opposite ends of the colony indicated that the threshold change was confined to the stem and branches of that part of the colony responding to the stimulation.

Fig. 3. A *Pennaria* colony following a shock of not much above threshold intensity applied to the stem at the point marked by the arrow.

The number of polyps which contract following a single near-threshold shock is quite variable. Increasing the stimulus intensity by 25 % above a shock which proved sub-threshold can cause contraction in from one to thirty polyps. Further increasing the intensity brings about greater contraction in those responding polyps which did not contract maximally, as well as causing more polyps to react. The effectiveness of increasing stimulus intensity is also quite variable. In over half the cases, increasing the stimulus intensity by 25 % more than doubled the number of responding polyps. This result was more likely, however, if few polyps had contracted at the lower intensity.

As Zoja (1891) found, the wave of polyp contraction usually spreads more easily toward the apex of the colony than toward the base. The number of responding branches is typically larger on the apical side of the electrodes. Since proximal branches bear more polyps than do distal ones, the difference in the number of polyps responding on either side of the electrodes is less marked, but usually more polyps contract distally to the electrodes than proximally.

A branch often acts as a behavioural unit; all the polyps on a responding branch generally contract to the same stimulus. Exceptions to this observation are seen, however, and occasionally only those polyps close to the main stem on a branch are affected. Infrequently the wave of polyp contraction skips one or a few polyps and these remain expanded while those on either side contract. In a similar way the

wave will occasionally miss an entire branch while polyps on branches on either side respond. This skipping of individual polyps or whole branches is usually seen only in the portion of a stimulated colony where polyp contraction is slow and incomplete.

(*c*) *Repetitive electric stimuli*

Although polyp contraction can be produced by a single shock, the threshold stimulus intensity is usually lower for repetitive stimuli and the number of polyps responding can be increased by stimulating one or more times following a first shock. The decrease in threshold intensity for repetitive stimuli is greater than that seen in *Cordylophora* and can be as much as 30%.

The frequency usually used for experiments with repeated stimuli was one shock per 5 sec. so that polyp responses caused by each stimulus could be fully evaluated. In over half the trials, excluding those cases where no polyps responded to the first stimulus, the increment in the number of polyps contracting due to the second stimulus was greater than the number responding to the first shock, although the results of any one experiment were variable and unpredictable. Usually only the first few stimuli of a series are effective in increasing the responding area. In a typical case, for example, six polyps contracted following the first stimulus, sixteen more responded to the second, and five additional polyps contracted after the third stimulus. The fourth, fifth and sixth shocks of this series caused contraction in no additional polyps. If an interposed shock is given at a shorter than usual interval after the stimuli of such a series have ceased being effective, a further spread of excitation can often be produced. Thus it is not only the number of stimuli at a given intensity but also the interval between them which limits the spread of polyp retraction.

With repeated shocks as with single stimuli, the degree of polyp contraction is greater near the electrodes and spread of excitation proceeds more readily distally than proximally.

If the electrodes are placed on a branch rather than on the main stem, a single shock typically causes contraction of only the polyps on that branch while an additional stimulus evokes contraction of polyps on several neighbouring branches. This is further evidence that branches often act as single responding units. The junction between the stem and a branch frequently presents a barrier to the spread of excitation which, once crossing this barrier, spreads more easily in the area beyond.

(*d*) *Conduction velocity*

As *Pennaria* colonies are long and the conduction velocity is quite slow, reasonably accurate conduction velocity measurements can be made with a stop-watch and visual observation. By using conduction velocity measurements, Horridge's (1957) second model of the functioning of nerve nets can be partially tested.

Horridge proposed that conduction in a nerve net may involve only a portion of the elements in the net, and that the density of these active units at any point in the net depends in part on the stimulating conditions; the more intense the stimulus the greater the density. If this is so, one should expect to find the conduction velocity a function of the stimulating conditions. Following a low-intensity stimulus, the excitation should spread along just a portion of the elements in the net, a portion determined by the field created by the electrodes and by the topology of the con-

ducting elements and the connexions between them. Such spread could follow either direct or meandering pathways. If these pathways are direct, the conduction velocity will be maximum; if indirect, something less. The average conduction velocity of a number of such experiments, then, should be less than the maximum conduction velocity which would be obtained if fortuitously the fastest and most direct routes were always taken by the excitation. If, on the other hand, most of the conducting elements available are stimulated, the conduction velocity measured will be that of the first-arriving impulses, those coming along the fastest and most direct pathways of all those available. Under such conditions the measured conduction velocity should always be the maximum of which the net is capable.

To see if the conduction velocity was a function of the method of stimulation, the conduction velocity was measured in *Pennaria* colonies while stimulating with a single shock or by a 1 sec. burst of forty shocks. It was felt that the latter stimulus would be sufficient to excite most of the available pathways in the colony and the conduction velocity following such stimulation would be maximal. The time between the beginning of contraction of polyps on opposite ends of the microscope field was measured with a stop-watch, and the distance between these polyps was measured with an ocular micrometer. This distance was usually about 1·5 cm. Only those cases in which the wave of excitation traversed the entire microscope field were counted. Over twenty conduction-velocity measurements were made with each method of stimulation. Usually each measurement was made with a fresh colony, although in some cases two or more measurements were made on the same colony. All experiments were done at 26° C.

The conduction velocity following a single shock was 1·04 cm./sec., while following a 1 sec. burst of forty stimuli it was 1·07 cm./sec. The difference between these averages is certainly less than could be accurately measured with this technique. A difference in the conduction velocity following these two methods of stimulation, then, either does not occur or is too small to be measured with this technique.

(3) Hydractinia aggregata *Fraser*

Gastropod shells inhabited by hermit crabs, dredged from shallow water near Friday Harbor, Washington, are often covered with colonies of *Hydractinia aggregata*. Such colonies resemble those of *H. echinata*, but lack spiral zooids.

(*a*) *Mechanical stimuli*

H. aggregata polyps are quite unresponsive to prodding with a glass rod, although occasionally several tentacles or the whole polyp will contract following such stimulation. Pinching a polyp causes it to contract, and often leads to contraction of from 1 to 200 neighbouring polyps. Pushing on or damaging the stoloniferous mat from which the polyps grow instigates a wave of contraction involving a large number of polyps, but usually not the whole colony.

(*b*) *Single electric stimuli*

A single near-threshold shock can elicit contraction in one or a few polyps near the electrodes. Increasing the stimulus intensity causes more polyps to respond; a 25% above-threshold shock usually evokes contraction of over 100 polyps and sometimes

causes contraction of all the visible polyps of the colony. Such polyp contraction spreads in a wave from the stimulating electrodes with an average velocity of 2·5 cm./sec. (15° C.). Polyps relax everywhere at the same rate following a near-threshold stimulus, taking 30–40 sec. to relax completely. At higher intensities, however, polyps near the electrodes relax more slowly than do those farther away, and often are not fully extended in 2 min.

(*c*) *Changes in sensitivity and responsiveness*

The sensitivity of *H. aggregata* colonies decreases steadily during a series of stimulations. In an experiment testing this threshold change, electrodes made of fine-tipped glass tubes filled with sea water were substituted for the usual insulated silver wires to avoid any electrode polarization. Electrical contact was made with the sea water in the electrodes by means of coils of silver wire covered with silver chloride. The electrodes were held in position with their tips firmly against the basal mat of stolons. In a typical experiment, the threshold rose steadily during fifteen determinations, all within 40 min. The threshold at the end of this time was nearly ten times that at the beginning. Experiments with two pairs of electrodes on the colony indicated that the threshold change was confined to that part of the colony responding to the stimuli.

The responsiveness to mechanical stimulation also decreases during an experiment. A glass tube, drawn to a fine, rounded tip, was allowed to slide within a larger tube mounted so that the inner tube always struck the same point of a *Hydractinia* colony. After such stimulation, the inner tube was withdrawn to a fixed height and 2 min. were allowed before the colony was stimulated again. In a typical experiment, an average of fifty-six polyps responded to each of the first five stimuli, fifty-two to the second five, nine to the third five, and an average of only five polyps responded to each of the last five stimuli. The water temperature was held at 15° C. throughout the course of both the above-described experiments.

(*d*) *Repetitive electric stimuli*

A second shock given shortly after an above-threshold stimulus increases the number of responding polyps. The effectiveness of this second shock is quite variable. Using a pair of stimuli separated by a 2 sec. interval, in six out of ten trials the number of polyps contracting following the second shock was more than twice the number which had contracted to the first shock alone. The second shock of a pair was effective in increasing the number of responding polyps at intervals of up to 6 sec. Only the first two or three shocks of a series of stimuli increase the number of responding polyps; further shocks have no effect. Following such repetitive stimulation, polyps near the electrodes relax more slowly than do those near the periphery of the responding area.

Polyp contraction following either mechanical or electrical stimulation often occurs in patches. For example, a circle of polyps near the electrodes will contract following a single shock and, after a brief but noticeable delay, another adjacent, often concentric group of polyps will respond. Frequently one of a series of stimuli will not cause more polyps on the whole periphery to contract but only a group of polyps of variable size on one portion of the periphery.

(*e*) *Spontaneous polyp contraction*

Spontaneous activity following stimulation is more commonly seen in *H. aggregata* than in *H. echinata*. Several seconds after a stimulus, usually in an area near the stimulating electrodes, a single polyp or a group of polyps often suddenly contract again. A wave of contraction can start from such an area which affects many polyps, sometimes even more polyps than had contracted following the original stimulus and occasionally involving all the visible polyps of the colony. Spontaneous activity is especially common following strong or repeated stimuli when waves of polyp contraction often originate from many foci in the colony, and polyps continue partially relaxing and recontracting for many minutes. The same area can initiate several waves. Typically, following strong stimulation, the patches of polyps spontaneously contracting decrease in size and the interval between such contractions lengthens until the colony is again quiescent. Such spontaneous activity indicates repetitive firing in the inter-polyp conducting system.

IV. *Species with little polyp co-ordination*

(1) Tubularia *sp.* (*probably* crocea (*Agassiz*))

Experiment on this species were done at the Friday Harbor Marine Laboratory. The hydroids were collected from Puget Sound near Tacoma, Washington. The large hydranths have two circlets of tentacles; a distal row near the mouth with about thirty tentacles and a proximal row just beneath the gonophores containing slightly fewer tentacles. The stalks bearing the hydranths are several centimetres high and grow from a tangled mat of stolons. These stalks are occasionally branched. The hydroids from Washington are larger and have more tentacles than *Tubularia crocea* from Los Angeles, but otherwise these two forms appear similar and may be the same species.

Tentacles in both the proximal and distal rows show much spontaneity; individual tentacles or groups of tentacles in the proximal row frequently move toward the mouth and tentacles in the distal row move away from the mouth. When a portion of the stalk is pinched with forceps or electrically stimulated, all the tentacles of the distal row are simultaneously moved away from the mouth (Fig. 4), a reaction similar to that described for *T. mesembryanthemum* by Zoja (1891). A single shock is sufficient to elicit some response, but the degree of tentacle opening is increased by stimulating again before the tentacles return to their resting position.

Pearse (1906) described several responses seen following mechanical stimulation of *T. crocea* polyps. These included folding of the proximal tentacles about the hydranth, folding of both proximal and distal tentacles about the hydranth, and opening of the distal tentacles. All these responses were seen among the spontaneous activities of the animals used in this study, but only the opening of the distal tentacles could be elicited by stimulating the stalk.

In some cases the opening of the distal tentacles can be produced only by stimulating the stalk near the polyp, but often stimuli applied anywhere on the stalk are equally effective in causing this response. In one case, for example, almost identical stimulus strength–duration curves were obtained when the electrodes were placed 3 mm. below the hydranth and when they were 2 cm. from the hydranth. The chronaxie for electrical stimulation is about 0·4 msec. The latent period between a just supra-

threshold stimulus and the beginning of the tentacle response gives a minimum conduction velocity of 3·5 cm./sec. (17° C.) without allowing for delay between the arrival of excitation at the polyp and the beginning of the tentacle response.

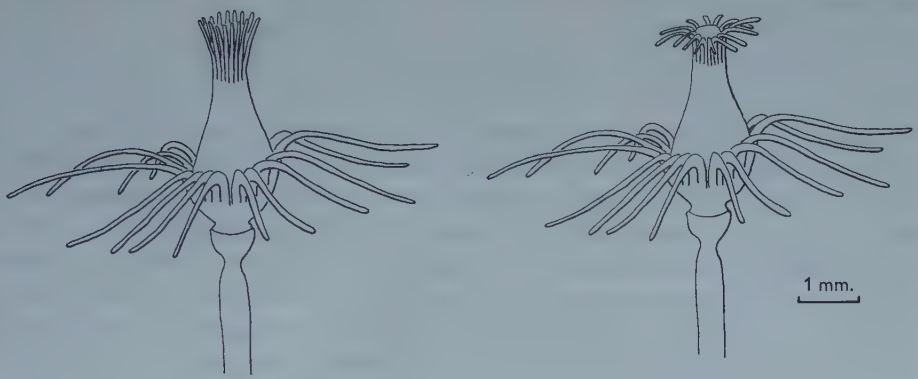

Fig. 4. A *Tubularia* polyp before and after stimulation of the stalk showing the characteristic opening of the distal tentacles.

In only one instance in approximately twenty-five attempts did more than one polyp respond to stimulation of a stalk, although in all cases the stalk stimulated was only one of many growing from a common mat of stolons. In the one successful attempt, the other polyp which responded was growing from a branch which left the stalk just proximal to the stimulating electrodes. The excitation produced by the stimulus must have proceeded in both directions in the stalk, distally to the main hydranth and proximally to the branch bearing the other responding polyp. Both polyps opened their distal tentacles. Other attempts to stimulate a stalk with a side branch failed to elicit responses from the polyp on the branch. Colonial co-ordination, therefore, seems poorly developed in *Tubularia*.

Often the stolons of a colony are transparent and appear devoid of living tissue. Jickeli (1883*b*) described a similar phenomenon in *T. mesembryanthemum*, where the coenosarc in the stem degenerates proximal to the hydranth and older members of a colony are joined only by empty tubes of perisarc. Such tissue changes may be the basis for the lack of behavioural communication between individuals in *Tubularia* colonies.

(2) Syncoryne mirabilis (*Agassiz*)

Syncoryne colonies are found growing under overhanging portions of rocks near the low tide level at Friday Harbor, Washington. These colonies consist of bushy tufts of stalks and stolons, each tuft containing several hundred polyps.

(*a*) *Mechanical stimulation*

Mechanical displacement which is abrupt although quite weak, when applied to the hydranth or merely in the water near the hydranth, causes immediate bending of the polyp toward the source of disturbance (Josephson, 1961). If the hydranth is carefully touched with a glass needle, it bends toward the needle. The angle to which the polyp bends increases with increasing stimulus intensity, as does the time taken by the polyp

to return to its resting position. This relaxation time can vary from a few seconds to over 1 min. Strong stimulation of the polyp or pinching the stalk bearing the polyp evokes contraction of the hydranth. The polyps are about 1·5 mm. high and can shorten in contracting by 30%.

(*b*) *Electrical stimuli*

A single electric shock to the stalk near the polyp can evoke either polyp bending or contraction. Stimuli just above threshold intensity cause polyp bending; stimuli much above threshold intensity (15–60% above threshold, dependent on the preparation) evoke polyp contraction. Often such responses can only be produced when the electrodes are no more than a few millimetres from the hydranth although on one occasion stimuli applied through electrodes 1 cm. down the stalk from the hydranth were effective. While a single shock can cause polyp bending, the degree to which a polyp bends and the time taken by it to relax can be increased by stimulating again before it has returned to its resting position. The chronaxie for electrical stimuli applied to the stalk is about 0·3 msec.

To investigate colonial co-ordination, hydranth-bearing stalks which had obvious tissue connexions with at least one other hydranth were stimulated. In only one of over twenty such experiments did more than just the hydranth on the stalk respond. In the one exception, a polyp on a branch leaving the main stalk proximal to the stimulating electrodes also responded by bending to each stimulus. In *Syncoryne* as in *Tubularia*, there appears to be little colonial co-ordination.

In one experiment, during the determination of a stimulus strength–duration curve, the threshold suddenly increased enormously. Upon examination it was noted that the coenosarc near the stimulating electrodes had parted leaving a 1·2 mm. length of empty perisarc. No strong stimuli had been given to the stalk; all the preceding shocks had been at most just threshold for the bending response. It appears that under some circumstances tissue in the coenosarc can contract, even though both Citron (1902) and Schulze (1873) failed to find muscular tissue in the coenosarc of *Syncoryne*. Such contraction may be related to the extremely slow pulsations seen in the coenosarc just behind the growing stolon tip in *Obelia* (Berrill, 1949) and *Clytia* (Hale, 1960), where again there is apparently no differentiated muscle tissue.

Thecate hydroids

Withdrawal of several polyps following damage to a single polyp or crushing of a stem has been seen in a number of thecate hydroids including *Obelia* spp. Because of their usual small size, the thecates are not as favourable experimental animals as many of the athecates, and the details of the conduction in this group have yet to be determined.

DISCUSSION

Colonial co-ordination in hydroids

The stems or stolons of all the hydroid species studied have systems capable of conducting excitation. This confirms the early observations of Zoja (1891), who similarly found spread of excitation in a number of different hydroids. In not all cases, however, is there colonial co-ordination. The frequency of occurrence of inter-polyp

behavioural communication in both *Tubularia* and *Syncoryne* is quite low. This may be due to tissue changes in the older parts of the stems of these species. In this respect it is interesting that conduction in *Pennaria* is polarized, progressing more readily distally toward the younger portions of the colony than proximally into the older coenosarc. Excluding *Tubularia* and *Syncoryne*, the hydroid species studied are similar in that they form structurally well-organized colonies, and all have systems capable of co-ordinating polyp contraction.

Evidence favours the presence of two inter-polyp conducting systems in *Hydractinia echinata*, one controlling local and the other colony-wide responses. These systems are distinguished by the different areas of polyps affected by activity in each, the greater conduction velocity of the through-conducting system as opposed to the local system, and by the different responses of the spiral zooids, becoming more tightly coiled in areas of local activity and lashing during colony-wide responses. The different activities of the spiral zooids could also be explained by multiple firing in the conduction pathways coupled with muscle systems in the polyp having different facilitation requirements. The unitary and all-or-none action of the spiral zooids, however, one lash to one shock, rules against this interpretation. In *H. echinata* the two conducting systems have nearly the same threshold for electrical stimuli. In those cases where the through-conducting system has the lower threshold, evidence of local activity is masked by the colony-wide response. The fact that through-conduction frequently has a lower threshold than local response further indicates two conducting systems rather than different types of activity, such as single impulses *v.* repetitive firing, in a single conducting system. If there are similarly two conducting systems in *Podocoryne*, the through-conducting system always had the lower threshold in the colonies investigated. The delayed relaxation of polyps near the electrodes possibly could be explained on the basis of a local system whose effects sum with those of the through-conducted response.

Conduction in coelenterates

The conducting systems of the hydroids investigated fall into one of two functional types: those which are through-conducting, have a sharp, rather constant threshold, are all-or-none with respect to stimulus intensity, and which do not show decreasing response with increasing distance from the point of stimulation; and those which are local, have no sharp threshold, show dependency of response on stimulus intensity, have responses which decrease in magnitude with greater distance from the stimulated point, are quite variable with regard to distance of excitation spread, and which are labile, the sensitivity and responsiveness decreasing with repeated use. Conduction in the stems of *Syncoryne* and *Tubularia* and the through-conducting systems of *H. echinata* and *Podocoryne* fall into the first category; the inter-polyp communication in the other hydroids studied and probably the local response of *H. echinata* fall into the second. Horridge (1957) found analogous local systems in the perforate corals *Goniopora* and *Porites*.

Through-conducting systems are found widely in the coelenterates, controlling luminescence and polyp retraction in *Renilla* (Parker, 1920*b*) and beating of the bells of jellyfish (Bullock, 1943), for example. Such systems can be explained on the basis of a nerve net in which all or almost all of the neurons are joined by synapses trans-

mitting each arriving impulse. The local systems described above, however, are not so easily explained.

In discussing the colonial responses of coral polyps, Horridge (1957) proposed two models, the first mechanical, the second mathematical. The mathematical model deals with the effects of stimulating a variable number of conducting units out of a larger population of such units. These units are able to excite one another, but the probability of activity in one unit initiating activity in another is, on the average, less than one. The mechanical model, discarded in part because of its high variability, represents a special case of the mathematical model, that in which only one unit is initially stimulated. Using the second model, Horridge was able to explain the lesser contraction of polyps toward the periphery of the responding area following a single shock on the basis of decreasing density of active units with increasing distance from the stimulating electrodes.

The colonial responses of both coral and hydroid polyps are usually symmetrical. Discussing the conducting system within each polyp controlling such responses, Horridge points out that: 'It may be inferred that this system acts in a through-conducting manner, effectively as a single nerve (fiber).' Such a system may be expected to be all-or-none, and therefore a poor device for differentially responding to changes in the density of active units in its vicinity. A single impulse in a through-conducting system might be just as effective as many simultaneously arriving impulses. Such a system could, however, respond differentially to a temporal pattern of impulses, provided that these impulses are at longer intervals than the refractory period of the conducting system. It could be argued that, because of the existence of fast and slow pathways in a nerve net, a greater density of active units also means a longer period of activity, and polyps are responding to repetitively arriving impulses associated with greater density of active units and not to the density *per se*. Polyps near the electrodes contract to a wave of excitation before it has travelled far and before impulses have had a chance to become spatially and temporally separated because of different conduction velocities. If it were a temporal pattern of activity associated with density which led to greater polyp contraction, polyps near the electrodes should contract less than those a greater distance away. This, however, is not the case. Postulated changes in the density of active units, then, would not seem to account for the observed lessening of polyp contraction with greater distance from the electrodes.

The properties of the local systems in hydroids which need to be explained are: (1) the dependency of distance of excitation spread on electrical stimulus strength; (2) the greater responses of polyps near the point of stimulation than those farther away; (3) the rapid decline of sensitivity and responsiveness; and (4) the inherent variability of these systems. Horridge's second model has been shown to be inadequate to explain (2) and, further, the change in conduction velocity with different stimulating conditions one expects from this model was not found in experiments on *Pennaria*. The possibility that changes in conduction velocity occur but were below the limit of detection cannot, however, be excluded.

Greater excitation spreads with increasing mechanical stimulus strength and greater responses near the area mechanically stimulated—properties once thought due to decremental conduction in nerve nets—were convincingly explained by Pantin (1935) on the basis of repetitive firing and interneural facilitation. It will be shown in the following paper that exactly the same explanation may be applied to electrical stimulation in

hydroids and possibly also for some corals. It may be pointed out here that there is behavioural evidence for repetitive activity following electrical stimulation in hydroids. The spontaneous activity often seen following stimulation in *Hydractinia* is certainly an example, and the patchy polyp contraction seen following a single shock in this genus can also be construed in this way. The variability of local responses in hydroids can be explained on the basis of a non-linear relation between stimulus strength and repetitive firing, and of different tendencies to repetitive activity in different preparations or in the same preparation at different times. The decrease in sensitivity and responsiveness is probably due to a decreased tendency to repetitive activity, although changes in the effective conductive path lengths because of changes in interneural junction properties may also be involved.

Responses of individual hydroid polyps

The conducting systems in the stems or stolons of hydroids usually initiate symmetrical polyp responses: contraction of the hydranth and/or simultaneous movements of the tentacles. The spiral zooids of *Hydractinia* and *Podocoryne*, however, always show asymmetrical responses and the polyps of *Syncoryne* bend to one side following just supra-threshold stimulation of the stalk. To account for the rapid bending of the latter species, it has been postulated that the longitudinal musculature of the hydranth is divided into parallel fields which can contract independently (Josephson, 1961). It seems likely that conduction in the stem of *Syncoryne* is predominantly longitudinal as Parker (1917) has shown it to be in the stalk of another hydroid, *Corymorpha*. The asymmetrical response of *Syncoryne* can then be explained as due to just supra-threshold shocks exciting only one of a number of parallel, longitudinal conducting pathways, and the induced excitation travelling up one side of the stem and exciting just one portion of the longitudinal musculature of the hydranth.

Stimulating the stalk of *Tubularia* initiates opening of the distal tentacles, indicating the presence of conducting pathways from the stalk through the hydranth to this circle of tentacles. The spontaneous symmetrical elevation of the proximal tentacles is evidence for the presence of a conducting system able to co-ordinate the activities of these tentacles. Thus the polyps of *Tubularia* probably have two concentric conducting systems between which there is no evidence of interaction.

SUMMARY

1. Conduction of excitation in response to local mechanical or electrical stimulation has been studied in various hydroid species.

2. There are systems in the coenosarc of the stems and stolons of all species which conduct excitation at rates of from 1 to 3·5 cm./sec.

3. Two physiological types of conducting systems have been found.

(*a*) Through-conducting systems, showing: all-or-none response, sharp threshold, reproducibility.

(*b*) Local systems, showing: spread of response dependent upon stimulus strength, no sharp threshold, responses which decline with increasing distance from the stimulated point, variability.

4. Colonial co-ordination is better developed in those species whose colonies are

structurally better developed. It is effected in most species by either (*a*) or (*b*). Both types are present together in *Hydractinia echinata*.

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REPETITIVE POTENTIALS FOLLOWING BRIEF ELECTRIC STIMULI IN A HYDROID

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INTRODUCTION

Only recently have electrical correlates of conduction in coelenterates been recorded (Horridge, 1954; Yamashita, 1957; Passano, 1958; Passano & McCullough, 1960). But before that, such progress had been made with indirect methods that Pantin, the leading student of this group, was able to state: 'Though its electrical concomitant has not yet been detected, the existence of the "all-or-nothing" impulse is as clear in coelenterates as in vertebrates' (Pantin, 1952).

The properties of the coelenterate nerve net that had been supposed to indicate decremental conduction—that is, increased distance of excitation spread with stronger mechanical stimuli and greater responses near the stimulated area than at some distance—were convincingly explained by Pantin (1935*a*) on the basis of repetitive impulses engendered by the mechanical stimulus and of interneural facilitation in the nerve net. Pantin found no evidence for decremental spread following electric shocks in the sea anemone *Calliactis*. Using muscle response as an indicator, he demonstrated that an electric shock in this species initiates a single all-or-nothing event in the nerve net.

Recently, however, distance of spread of excitation varying with the stimulus strength and intensity of response varying with the distance from the point of stimulation have been found following single, brief, *electrical* stimuli in colonial corals (Horridge, 1957) and hydroids (Josephson, 1961). This paper will present evidence from electrical recordings that such responses, at least in hydroids, are due to repetitive impulses in the conducting system, even following brief electric shocks which are not much above threshold intensity.

MATERIALS AND METHODS

The species used in this study was the gymnoblastic hydroid *Cordylophora lacustris* Allman. Hydroid colonies were grown in sea water diluted to 10% by aged water from a freshwater aquarium. The temperature of the cultures was about 21° C. Several times a week the animals were fed on newly hatched brine shrimp. Under these conditions *Cordylophora* forms colonies consisting of a single stolon, firmly attached to the substrate, and, at 2–5 mm. intervals, short, upright stalks, each bearing a single hydranth. The stolons are 0·13 mm. in diameter; the stalks bearing the hydranths and the hydranths themselves are each about 1 mm. high. The older polyps and the coenosarc regress as new polyps form, and a complete colony grown under such conditions contains only three to six hydranths.

Electric potentials were recorded from the stolons of *Cordylophora* by stainless steel micro-electrodes etched and insulated as described by Green (1958). The electrodes used had tip diameters of from 1 to 3 μ. Potentials were recorded between one or two micro-electrodes and an indifferent silver electrode in the solution surrounding the polyp. Conventional capacitor-coupled amplifying and recording equipment was used.

Electric stimuli were delivered to the colonies through fine silver wires (0·2 mm. in diameter) insulated to the tip and placed one on each side of the stolon (Fig. 1). The stimuli were square pulses produced by a Grass S4 stimulator and were, unless otherwise stated, 0·5 msec. in duration.

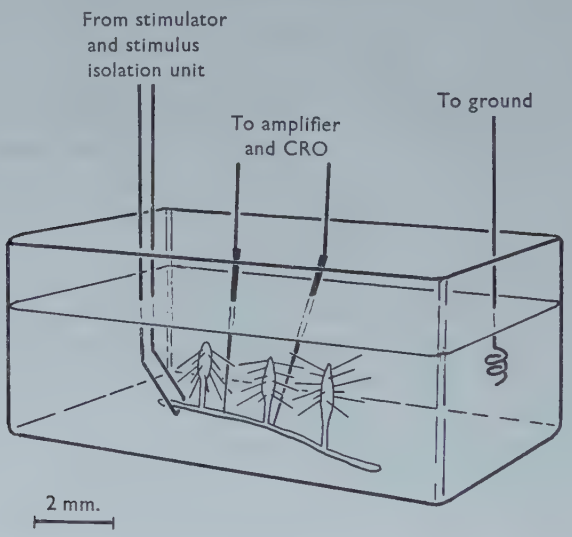

Fig. 1. The method of recording. The hydroid colony is shown as it might appear following a stimulus of not much above threshold intensity; the polyp nearest the stimulating electrodes is partially contracted, the next polyp is somewhat less contracted, and the most distal polyp is unaffected.

Five minutes were allowed between each stimulus or group of stimuli constituting a single test. Only with such long intervals between trials can repeatable results be obtained. The threshold continually rises and the characteristic tendency to repetitive firing declines with stimulus frequencies as low as one per minute. All experiments were done on colonies completely submerged in dilute sea water. If only a few stimuli are given at each trial, the colonies show no signs of deterioration during the course of an experiment, and measurements can be made for many hours.

RESULTS

Large negative potentials, 0·05–15 mV. in amplitude, can easily be recorded from an electrode impaling the stolon of *Cordylophora*. These potentials are not spontaneous; they are only seen following stimulation. The amplitude of the potentials depends as much upon the particular electrode used as on any other factor. Small changes in the position of the recording electrode cause little alteration of the measured response. There is considerable variation in the duration of the potentials; they can be as short as 20 msec. or as long as 120 msec. Similar potentials can also be recorded from an

electrode inserted in the wall of the hydroid body. The flexible hydranth, however, is more difficult to impale than the rigid stolon, and the latter was the preferred location for measurement in this study. Since the potentials recorded are not yet known to be equivalent to nerve action potentials, the terms 'impulse' or 'spike' will not be applied. They will be called 'pulses' in this discussion.

A train of pulses can follow a single shock of less than $1\frac{1}{2}$ times threshold intensity (Fig. 2). As many as twelve pulses in a burst lasting several seconds can be produced

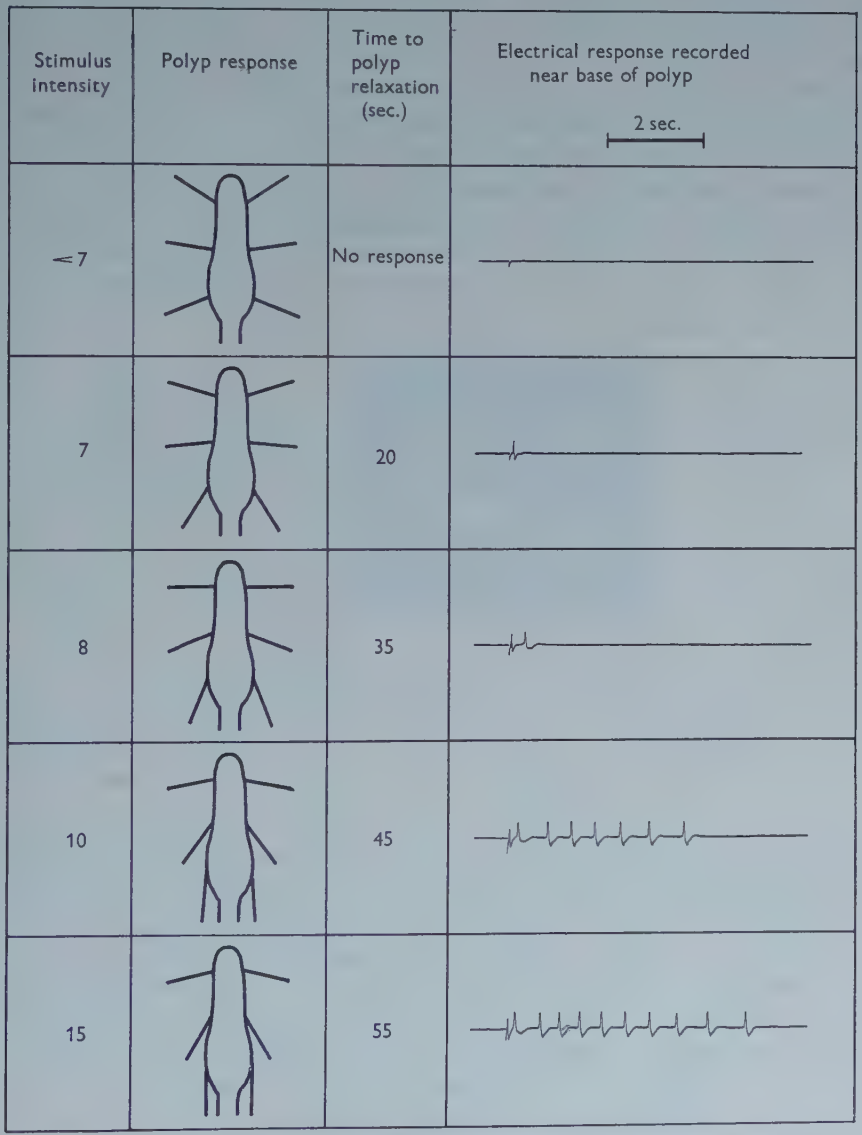

Fig. 2. Comparison of the response of a polyp and the electrical potentials recorded in the stolon near the polyp stalk. Only tentacle depression is shown in the diagrams of the responding polyp; hydranth shortening, which also occurs, is not shown. The times to relaxation are approximate since the end point (complete relaxation) is difficult to determine exactly. The deflection in the first electrical record is the stimulus artifact.

by a single 0·5 msec. stimulus. The shape of the pulses in such a series is quite characteristic. The first is short, 20–40 msec., and is usually the smallest in amplitude of the series. It tends to be more diphasic than any of the others, often being followed by a marked positive potential. The second pulse follows the first after an interval of about 200 msec. It is usually the longest pulse of the series. The remaining pulses are all similar in shape and height, usually having a form intermediate between that of the first two pulses, being higher and longer than the first and more diphasic than the second. They follow one another at increasing intervals. The interval between the second and third pulse is usually longer than that between any except the last few pulses of the train.

The number of pulses in a burst, but not the shape of any single pulse, varies with the stimulus strength. The threshold for single pulses is sharp (Fig. 3). A just supra-threshold shock initiates a single pulse, a stimulus $1\frac{1}{3}$ times threshold frequently gives a short burst, and stronger stimuli can evoke longer bursts.

Similar potentials have also been recorded from the stolon of the hydroid *Tubularia crocea* during the conduction of excitation. Unlike *Cordylophora*, little tendency to repetitive firing was seen in the few experiments done on this species.

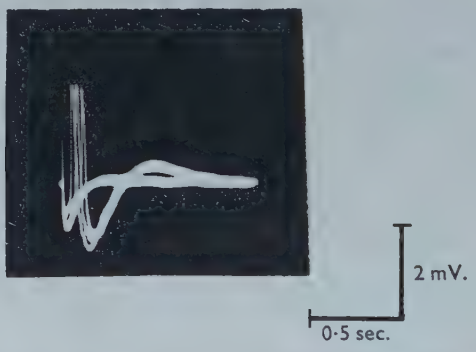

Fig. 3. Five superimposed sweeps taken 5 min. apart showing the sharp threshold and all-or-none nature of single pulses. The stimulus intensities were 8, 9, 10, 11 and 12.

Correlation between electrical activity and polyp response

When the stolon of a *Cordylophora* colony is stimulated, a wave of polyp contraction is initiated which involves some or all of the polyps of the colony. The tentacles are all depressed and the hydranth body shortened in a contracting polyp. The number of polyps responding, i.e. the distance of spread, is a function of the stimulus strength. With a just supra-threshold shock, one to three polyps near the electrodes contract. Increasing the stimulus strength causes more polyps to contract, and also leads to greater contraction in the polyps which previously responded. A second shock shortly following an effective one can increase the number of responding polyps and the degree of polyp contraction in the already active polyps.

The electrical activity measured in the stolon near the junction of the stolon and a stalk is exactly correlated with the behavioural response seen in the polyp on the stalk (Fig. 2). The larger the number of pulses recorded, the faster is the depression of the tentacles, the greater is the depression, and the longer is the period until relaxation is

complete. The polyp contraction is slow (taking several seconds) and smooth; it gives no hint of underlying repetitive activity.

Polyp responses are often graded with distance from the stimulating electrodes. The pulses, however, are not conducted with decrement. They are similar in height and shape wherever the colony is stimulated. When recording is made simultaneously from two electrodes separated by some distance in a long colony, the mechanism of graded response becomes apparent. The number of pulses seen at the two recording sites is often different; the electrode more removed from the point of stimulation records fewer pulses than does the electrode near the stimulated area. Polyps far from the stimulating electrodes, then, receive and respond to fewer pulses than do those near the point of stimulation. When the conduction velocity through the stolon is considered, it can be shown that it is the first pulses recorded in the electrodes nearer the point of stimulation which do not reach the distal electrode. The first pulses are not transmitted the length of the colony, but do pave the way for future pulses, a process suggestive of the interneural facilitation described by Pantin (1935*a*) from the oral disk of *Calliactis*.

Facilitation in the distance of excitation-spread can also be demonstrated with repetitive stimuli. In one experiment, for example, three shocks were given to the stolon at 1 sec. intervals. Two pulses were recorded from an electrode near the stimulated area following the first stimulus and one pulse was recorded following each of the next stimuli. A more distal electrode, however, recorded only one pulse, that following the third stimulus. The three pulses created by the first two stimuli did not reach the distal electrode, while the fourth pulse did.

Frequently two or three adjacent polyps form a behavioural unit; they all contract to the same degree following a stimulus and no one polyp of the group can be made to respond alone. If two electrodes record simultaneously from different places in a group of polyps, similar potentials are seen from each. Often all the polyps of a small colony form a single behavioural unit and potentials recorded anywhere in the colony are similar. It is only with electrodes widely separated in long colonies containing at least four polyps that facilitation of the distance of spread can be predictably demonstrated.

Characteristics of the pulses

(1) *Conduction velocity*

By measuring the latency between the appearance of pulses at two recording electrodes, the conduction velocity in the stolon of *Cordylophora* is shown to be 2·7 cm./sec. (average of ten measurements, range from 2·1 to 3·3 cm./sec., 22° C.). A similar figure is obtained by measuring the distance between the stimulating electrodes and a single recording electrode, and dividing by the latency between a just supra-threshold shock and the appearance of the pulse at the recording site. This indicates that the pulses originate in the stimulated area. The conduction velocity is constant for each pulse of the series, indicating a common conduction pathway.

(2) *Refractory period*

Attempts to measure the refractory period of the conducting system were made by giving a pair of shocks to the stolon separated by varying intervals. If the intensity was just supra-threshold, a single shock caused one pulse, and a pair of shocks, usually

only when separated by a sufficient interval, produced two pulses. The sufficient interval proved quite variable, and ranged from 12·5 to 700 msec. in different preparations. Since both stimuli were just above threshold, this would seem to be a measure of the relative refractory period. In one preparation, however, two shocks produced two pulses even with a vanishingly short interval between stimuli. This was verified in this preparation repeatedly. To investigate very short intervals, stimuli of 0·1 msec. duration were used. When the intensity was adjusted so that one shock gave one pulse, two shocks separated by any interval from 500 to 0·2 msec. consistently produced two pulses. Shorter intervals than 0·2 msec. could not be used with the equipment available. It would appear that the refractory period of the stolon of *Cordylophora* is exceedingly short or, more probable, non-existent, and that the minimum effective interval between two shocks seen in other preparations was a measure of something other than the refractory period. The lack of a refractory period and its significance for an understanding of the repetitive firing are discussed below.

If the interval between a pair of just supra-threshold shocks is greater than about 200 msec., the interval between the induced pulses is the same as the interval between the stimuli. If the interval between the stimuli is less than 200 msec., the pulses are always separated by about 200 msec. In the preparation mentioned above, even two stimuli 0·2 msec. apart evoked two pulses separated by 200–250 msec.

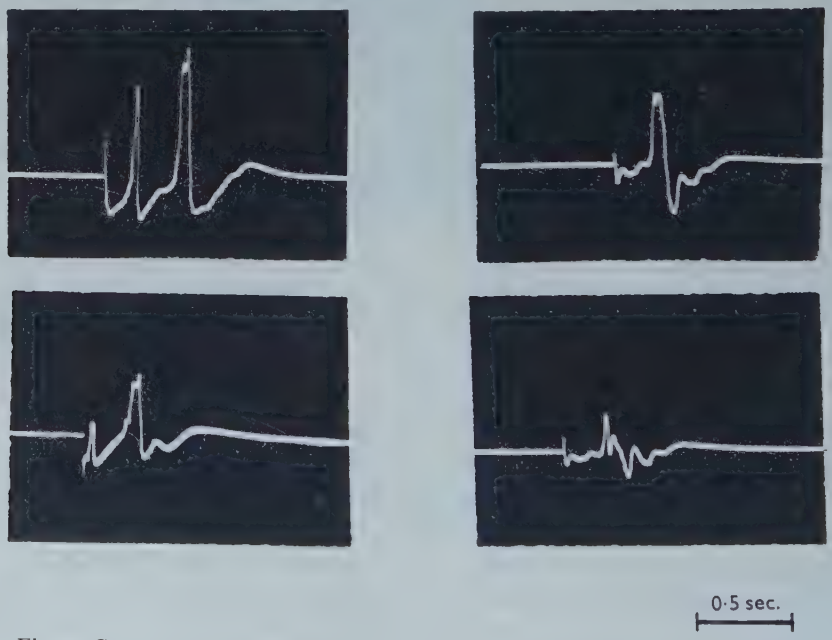

Fig. 4. Some atypical potentials which show the compound nature of the pulses.

(3) *The form of the pulse*

Although pulses are usually smooth in shape, potentials are occasionally recorded which reveal their compound nature (Fig. 4). On one occasion the first pulse of a train was a small asynchronous burst of potentials. These small potentials gradually coalesced so that the ninth and final pulse of the burst resembled those typically seen.

Sometimes the single pulses following just supra-threshold shocks become more ragged with repetitive stimulation, but more commonly they retain their smooth form.

The shape changes seen in successive pulses in a burst are also seen in single pulses following repetitive stimulation. For example, if two just supra-threshold shocks are given to the stolon at a 1 sec. interval, the second pulse recorded is less diphasic than the first and is longer in duration—exactly the same changes as seen between the first two pulses of a burst following a single stronger stimulus. The change in the shape of the pulse following a second shock is not all-or-none, but appears gradually with decreasing intervals between the shocks, first being noticeable at an interval of about 5 sec. The different shapes of pulses in a burst, then, do not necessarily indicate different conducting systems, but more probably changes in the properties of a single conducting system.

(4) *Interaction between pulses*

Two pulses started from stimulating electrodes at opposite ends of a colony cancel one another where they meet and only the first pulse reaching the recording electrode is seen. If the pulses are timed so that they meet at the recording electrode, the amplitude of the recorded potential is not greater than that of a single pulse. On the contrary, it is somewhat smaller, although of a longer duration than the single pulses. This indicates that no new elements are contributing to the potentials during conduction in different directions. The lack of summation of two pulses where they meet and their mutual cancelling show that conduction in different directions probably involves the same conducting elements.

(5) *Facilitation of pulse height and number*

The amplitude of single pulses following just supra-threshold stimuli can usually be increased by repetitive stimulation. A change in the height of the second pulse is often seen with a pair of stimuli separated by as much as 10 sec. The facilitation of pulse amplitude reaches a maximum at stimulus intervals of 1 sec., and does not change with still smaller intervals between shocks. The increase in pulse amplitude following repetitive stimulation can be as much as 70%. Often, however, little or no facilitation can be demonstrated, and infrequently a second pulse is smaller than the first. The change in the amplitude of the first two pulses of a burst is probably due to the same facilitation that affects single pulses with repetitive stimuli. Similarly, in a burst the second pulse is usually higher than the first, although occasionally the two pulses are equal in height and atypically the second pulse is smaller than the first.

The number of pulses following a stimulus can be increased by repetitive stimulation. In three colonies in which this was examined with paired shocks, the facilitation as measured by the greater number of pulses produced by the second shock was maximal at stimulus intervals of 1 sec. The facilitation declined on either side of this value, and was not detected at stimulus intervals of 0·1 or 10 sec. It is interesting to note that facilitation of pulse number was not seen with just supra-threshold stimuli. As was stated above, a pair of such stimuli produce just two pulses.

Facilitation of pulse number is only seen with the first two or three stimuli of a series. After the first few shocks, the number of pulses created by each shock steadily declines (Fig. 5). Even single pulses cannot be obtained after seven to ten shocks when

using stimulus frequencies of 1/sec. or 5/sec. Occasionally the pulses of a very long burst evoked by strong repeated stimuli decline in amplitude toward the end of a train, but more often they retain the same height throughout the course of a burst.

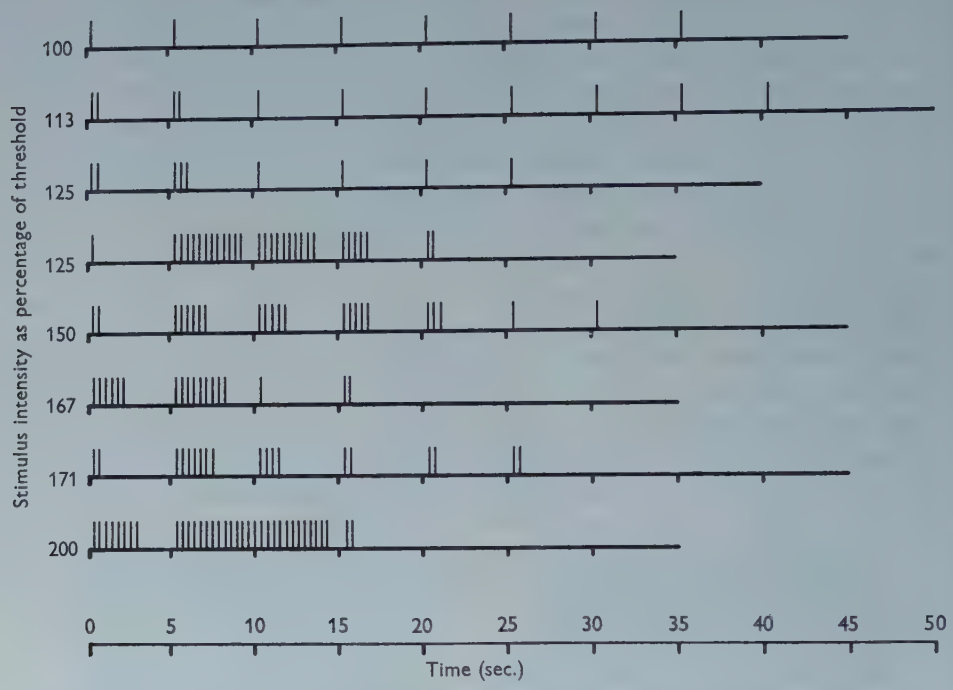

Fig. 5. The number of pulses initiated by each shock of a series at 5 sec. intervals. These are typical examples, and are taken from experiments done on several different colonies. The short bars below each line indicate shocks, the bars above each line indicate the pulses evoked.

Mechanical stimulation

Cordylophora colonies are quite sensitive to prodding of the stolon. Such stimulation usually leads to contraction of all the members of the colony. Pulses identical in form to those seen following electrical stimuli are recorded from the stolon following mechanical stimulation (Fig. 6). Gentle prodding of the stolon produces one or two pulses, more forceful prodding produces a burst of pulses. Prodding of the polyp usually initiates no electrical response in the stolon (nor does it cause contraction of neighbouring polyps) but pinching a polyp with forceps produces a burst of pulses and contraction of the polyps of the colony.

The site originating the bursts

One would expect the greatest concentration of nervous elements in a hydroid to be in the polyp body. The hydranth, therefore, falls under suspicion as the area which might initiate the repetitive activity recorded in *Cordylophora*. Removing all the hydranths from a colony, however, does not change the response seen in the stolon following stimulation. The stolon itself can give rise to a burst of potentials, and there seems to be no reason to believe it is not the stolon which normally initiates repetitive activity following stimuli applied to it.

In a few experiments involving simultaneous recording from two electrodes at increasing distances from the stimulating electrodes, evidence was found of pulses originating other than at the stimulated point. In these cases, following a pulse propagated in the usual direction, a second pulse appeared in the electrode more distal to the stimulating electrodes *before* it was seen at the recording electrode closer to the stimulated area. This pulse must have originated closer to the distal electrode than to the proximal one, and hence could not have arisen at the point of stimulation. Such multiple origin of pulses is not common; evidence for it was seen in only 3 out of over 100 simultaneous recordings from two electrodes.

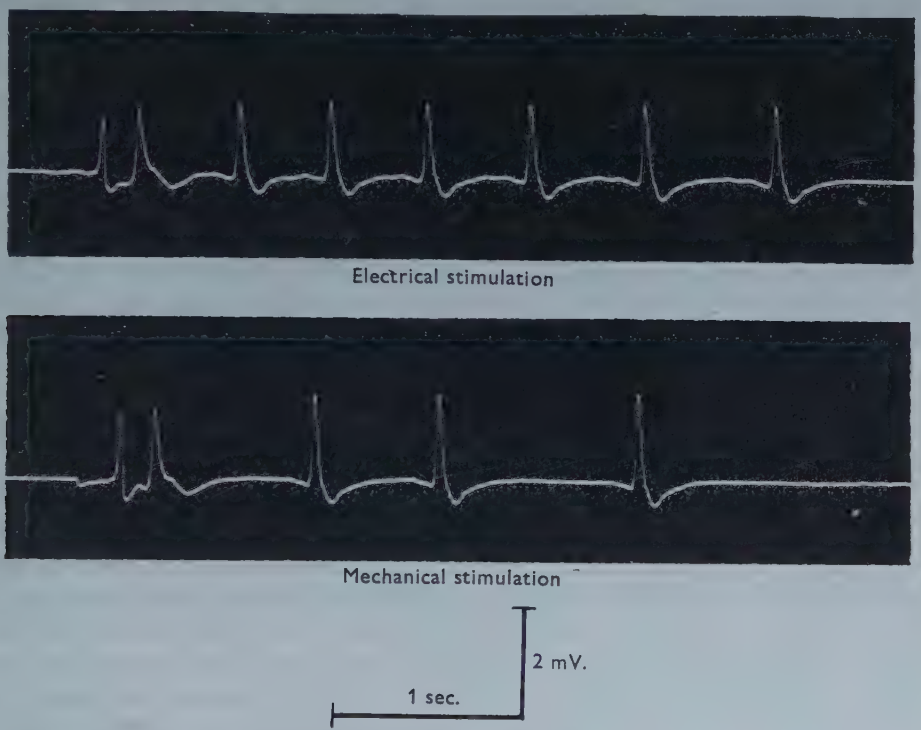

Fig. 6. Comparison of the recorded potentials following mechanical and electrical stimulation. The electrical stimulus was a single shock, 40% above threshold. The mechanical stimulus was a prod to the stolon. The positive deflexion of the base-line preceding the pulses in the lower record is probably a stimulus artifact.

Coenosarc movements during conduction

When the coenosarc of *Cordylophora* was observed with a magnification of 100 diameters, tissue movements were sometimes detected during the time a pulse was transmitted through the stolon. The movements usually consisted of a brief jerk, and involved displacement of certainly less than 15 μ. They were usually only seen following the first supra-threshold stimulus to a well rested colony. A second stimulus shortly after a first, although it often produced a longer train of pulses, usually did not cause discernible tissue movements. Although accurate measurements were not made, such tissue movements appear to be approximately temporally coincident with the passage of a pulse through the stolon.

Coenosarc movements could be due to active contraction of the observed area or to movements elsewhere in the colony mechanically transmitted through the stolon. They are not caused only by hydranth contraction, for they were also observed in colonies from which all the hydranths had been removed. Two observations on such hydranth-less colonies suggest that coenosarc movements may not be due to contraction of the area being observed. First, movements were often seen of only the fluid in the central space of the coenosarc and not of the tissue itself. Secondly, in one colony the movement seen was clearly an expansion of the coenosarc, which became more closely apposed to the outer perisarc. These observations indicate that contraction of the coenosarc of some area other than that being observed can cause a shift of fluid and an increase in the internal pressure of the stolon. Although this has not been investigated, the areas which come under suspicion are the terminal ends of the stolon, where the coenosarc is known to be contractile in some other hydroids (Berrill, 1949; Hale, 1960), or the cut ends of the polyp stalks.

Artifacts due to tissue movements do not appear to contribute to the recorded potentials. Pulses recorded when there was discernible movement near the electrode were identical to those recorded when no movement could be seen. When the wall of the stolon is displaced, there is produced a sudden, clearly observable shift of fluid and tissue in the stolon which is much greater than the movements described above. Such movements in the stolon can introduce artifacts in the electrical records (Fig. 6), but these are many times smaller than the pulses and do not interfere with measurements.

DISCUSSION

The significance of repetitive firing

The first point to be gained from this study is that while many conclusions can be drawn regarding conduction in coelenterates by the use of indirect methods, some properties of the conducting systems are elucidated only by electrical recordings. The muscular movements of *Cordylophora*, for example, are smooth and reveal no indication of underlying repetitive activity. Without direct measurements, one would suspect repetitive firing following single shocks only after eliminating other possibilities. Electrical measurements, however, make immediately evident the repetitive activity usually following stimulation. Analysis of conduction in *Cordylophora* by indirect methods is further complicated by the non-linear relation between stimulus strength and number of pulses initiated and by the changing effectiveness of shocks in a series, there being an early facilitation of pulse number followed by a decline in the number of pulses per shock (Fig. 5).

Some properties of excitation-spread following electrical stimulation in some colonial corals and hydroids are difficult to explain on the basis of Pantin's now classical scheme of nerve nets. These properties are: (1) the greater distance of spread with more intense single shocks; and (2) the greater response in polyps near the point of stimulation than in those more removed from the stimulated area. Horridge (1957), in discussing his observations on corals, suggested that these properties could be explained on the basis of a nerve net in which only a portion of the available elements in any area carry a nerve impulse when a wave of excitation passes. He postulated that the greater distance of spread with stronger stimulation was due to a larger number of

units being initially activated and hence a greater probability of the excitation finding long pathways in the net. The decrease in the magnitude of the polyp responses with greater distance was suggested to be correlated with a decrease in the density of active units with greater distance from the stimulated area.

It seems more probable on the basis of this and previous studies (Josephson, 1961) that the anomalous excitation-spread in some colonial coelenterates is due to repetitive activity, even following single shocks. This is certainly the case in *Cordylophora*. The increased distance of spread with stronger shocks finds its explanation in more pulses being initiated, and hence more barriers crossed by interneural facilitation (or its counterpart if spread in hydroids turns out to be by means of some other system than the nerve net, a proposition which now seems unlikely). The polyps near the stimulating electrodes respond more than those at some distance because they receive more pulses. Thus the explanation advanced by Pantin (1935*a*) for responses following mechanical stimulation also applies to responses following electrical stimulation in some species.

Repetitive firing following prolonged or very strong electrical stimuli is frequently encountered in animals. Even repetitive firing following brief electric shocks is not uncommon, and has been reported especially for crustacean nerves and giant-fibre systems in annelids (see, for example, Barnes, 1934; Bullock & Turner, 1950; Kao & Grundfest, 1956, 1957). Repetitive activity following prolonged stimulation is seen for luminescence in sea pens (Buck, 1953; Nicol, 1955, 1958; Davenport & Nicol, 1956) and for polyp contraction in some colonial hydroids (Josephson, 1961). Pantin (1935*b*) reported supernumerary contractions in some specimens of the sea anemone *Calliactis* following a battery of stimuli, indicating after-discharge in the nerve net, and Pantin & Vianna Dias (1952) reported a similar phenomenon in the jellyfish *Aurellia*. This after-discharge, at least in *Calliactis*, is unlike repetitive firing in *Cordylophora* in that it bears only a very indirect relation to the stimulus, both in time of appearance and in number of pulses. Despite this wide occurrence of repetitive firing in the Animal Kingdom, it was still surprising to find patterned bursts of output following stimulation, obviously an integrating mechanism, to be so well developed in such phylogenetically primitive and seemingly undifferentiated tissue as that of a hydroid stolon.

Conduction in hydroids

The potentials recorded from the stolon of *Cordylophora* are associated with the conduction of excitation. They may be created by the conducting system itself or only a reflexion of activity in the conducting system. They could, for example, be muscle potentials associated with conduction in a nerve net. There would seem to be two important problems related to the electrical correlates of conduction in a hydroid stolon: (1) the nature of the conducting system; and (2) the origin of the potentials.

(1) *The conducting system*

Some of the properties of conduction are best explained on the basis of a nerve net. The compound nature of the potentials indicates activity in a number of parallel channels. The failure of such potentials to become temporally dispersed with increasing distance from the point of stimulation, a phenomenon characteristic of nerve

trunks, is to be expected from a system like a nerve net with many lateral connexions. In such a system, the fastest conducting elements could continually excite neighbouring parallel fibres by cross-fibres joining them. The conduction velocity of the whole system, then, would be determined by the conduction velocity of its fastest elements. In the best case seen in this study demonstrating the compound nature of these potentials (described above) it was the early pulses of a burst which were dispersed. The coalescing of the small potentials into the more usually formed pulses at the end of the burst is interpreted as due to an increase in the effectiveness of the lateral connexions because of facilitation, with the result that for the later pulses the system fired as a nearly synchronous whole. An increase in the effectiveness of lateral connexions and perhaps recruitment of more conducting elements, both due to interneural facilitation, may be the basis of the changes in the shapes of pulses during a burst. And, in further support of nervous conduction, Mackie (1961) has recently found neurons histologically in the coenosarc of *Cordylophora*.

Muscle is the only tissue other than nerve generally credited with the ability to conduct excitation over some distance in metazoans. There is conflicting histological evidence for muscular tissue in hydroid stolons. Allman (1853), Schulze (1871, 1873), Citron (1902), Berrill (1949), and Hale (1960) failed to find muscle fibres in hydroid stolons; Hamann (1882) reported epithelial muscle cells in this region. The coenosarc of hydroids is capable of movement. Evidence for contractility has been seen in *Syncoryne* (Josephson, 1961), and a limited region of the coenosarc is contractile in *Obelia* (Berrill, 1949) and *Clytia* (Hale, 1960). The coenosarc of *Cordylophora* shows exceedingly slow shape changes, and small twitches are occasionally—but importantly not always—seen during the conduction of pulses. Muscular conduction through the stolon cannot be ruled out, but, because there is often conduction without any visible movement of coenosarc tissue, seems improbable.

The conduction velocity in *Cordylophora* is slower than one expects from nervous conduction. Conduction in a diffuse nerve net would involve many synaptic delays, so a slow conduction velocity is not totally unexpected.

(2) *The origin of the potentials*

The short duration of the potentials makes it seem likely that they are due to activity of nervous tissue. The separate potentials making up the compound pulse are probably shorter or, at a maximum, the same duration as the pulse. That they are shorter is indicated in some of the records showing the compound nature of the pulses, where evidence for potentials of quite short duration is occasionally seen (notice the short potential superimposed on the longer pulse in the first record of Fig. 4). Although it is unwise to speculate on the membrane potential *v.* muscular contraction relations in a phylum in which they have not yet been studied, a 20 msec. action potential seems quite fast for an animal whose contractions, as seen in the polyp, usually take several seconds to complete. The only clear recordings from single nerve fibres in coelenterates which have been published are those of Horridge (1954). As closely as I can measure from his figures, the action potentials from the large fibre system of *Aurellia* are about 8 msec. in duration, rather long when compared to those of other animal phyla and certainly not too short to be considered as components of the compound potentials described in this study.

The size of the potentials (up to 15 mV.) and the ease with which they can be recorded are not what one would expect from a diffuse nerve net. The stolons of hydroids may be close to ideal for electrical recording. Shunting would be minimized because of their small dimensions and by the surrounding cover of thick perisarc which may be electrically insulating. Recording from hydroid stolons might be equivalent to recording from a fine nerve completely immersed in oil. Potentials can also be easily recorded from the hydranths of *Cordylophora*, however, where these conditions do not prevail. The explanation for the large size of the pulses probably lies in the summing of simultaneous potentials from a number of elements in a system held in synchrony because of the activity of lateral connexions.

Although the evidence is far from incontrovertible, it seems to indicate that both conduction in *Cordylophora* stolons and the potentials associated with this conduction result from activity in a nerve net.

The initiation of repetitive firing

For a system to fire repeatedly following a single brief shock, it must have a memory. It must 'know' after it has fired once that it has yet to fire again. An axon with its all-or-none action potentials has no such memory. In firing and the subsequent restoration of the membrane it destroys evidence of the local responses which initiated the firing. Some cell bodies, synaptic areas, and sensory terminations have a memory. These areas often do not fire in an all-or-none fashion and can maintain a local depolarization capable of initiating spikes in some other part of the cell. Such areas are integrative in that they can sum activity, often both excitatory and inhibitory and from many inputs, over some time period (see Bullock, 1957, for a discussion of the role in integration of membranes which normally do not show propagated spikes). Since *Cordylophora* stolons show repetitive activity following brief shocks, it may be concluded that the area initiating the pulses (it need not be part of the conducting system itself) is like such integrative areas in that it has a memory, and therefore probably does not show all-or-none action potentials. One would not expect such an area to have a refractory period, and the inability in one case to demonstrate a refractory period in *Cordylophora* is in confirmation of the concept of a locus initiating the pulses which responds in a graded manner. The minimal interval between a pair of effective just supra-threshold stimuli more usually seen is probably due to decay with time of the excitation at the initiating area coupled with the refractory period of an all-or-none conducting system.

A minimal interval between successive potentials following progressively more closely spaced shocks has been reported several times for vertebrate nerves. It has been explained as due to: (1) the second potential travelling in an area left refractory by the activity of the first potential and having an initially slower conduction velocity (Gasser & Erlanger, 1925); or (2) a longer latency for the second potential because of a prolonged shock-response interval (Rosenblueth, Alanis & Mandoki, 1949). The similar phenomenon in *Cordylophora* may be due to yet another mechanism; a slow return in the sensitivity of the conducting system following firing, either directly or secondarily induced by the stimulus, until its threshold matches the intensity of a prolonged excitatory state maintained by an impulse-initiating area.

SUMMARY

1. Electrical pulses (amplitude −0·05 to −15 mV.; duration 20–120 msec.) have been recorded from the stolon of *Cordylophora lacustris* following stimulation. These pulses are propagated with an average velocity of 2·7 cm./sec. at 22° C.

2. Brief electric shocks of little more than threshold intensity can evoke bursts of pulses. The number of pulses in a burst increases with stimulus intensity, but the shape and size of individual pulses do not.

3. Repetitive stimulation causes facilitation of both size of single pulses and number of pulses in a burst. Refractory period, if present, is variable. The minimum interval between two pulses is about 200 msec.

4. Mechanical stimulation evokes pulses identical to those evoked by electrical stimulation.

5. The greater the number of pulses recorded in the stolon near a polyp, the greater and faster is the contraction of that polyp.

6. The number of pulses, but not their individual sizes, decreases with increasing distance from the point of stimulation.

7. It is concluded that conduction in the stolon and the electrical pulses are due to nervous activity and that the conducting system is a network having interneural junctions which sometimes require to be facilitated.

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THE ANNUAL GROWTH-RATE CYCLE IN BROWN TROUT (*SALMO TRUTTA* LINN.) AND ITS CAUSE

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INTRODUCTION

Three-year-old brown trout living in a hatchery stewpond have a growth rate which fluctuates in a regular annual cycle (Swift, 1955). The cause of these fluctuations is of interest in a study of the physiology of growth in trout. It was therefore important to establish whether these fluctuations are only found in the growth rate of fish living in a hatchery, and also whether the form of the cycle is the same in immature as in mature fish. If this form of annual growth-rate cycle is common to both mature and immature fish living both in the wild and in the hatchery, then the causes of the cycle should be ascertained.

The work undertaken is described in two parts. In the first part the growth-rate cycle of yearling trout living wild and in the hatchery is described, and its relationship with the two major variables in the external environment, water temperature and the daily length of the photoperiod, is discussed.

The second part of the work deals with the relationship between water temperature, photoperiod length and growth rate as established when the fish were grown in constant-environment aquaria.

PART I. THE ANNUAL GROWTH-RATE CYCLE OF YEARLING TROUT GROWING IN A HATCHERY STEWPOND AND IN THE WILD

Methods

For the examination of the growth rate under hatchery conditions a population of 100 yearling fish, selected at random from the hatchery stock, was kept in a concrete-lined stewpond holding approximately 25 kl. The fish were fed to satiation on minced liver. Each month the whole of the population was removed from the pond and the length of each fish, anaesthetized with tricaine methano-sulphonate, was recorded. From the means of these monthly measurements the average specific growth rate of the population was calculated, using the formula

$$\frac{\ln L_2 - \ln L_1}{t_2 - t_1} \times 100,$$

where $\ln L_1$ and $\ln L_2$ are the natural logarithms of the mean lengths of the populations at time t_1 and t_2 respectively, the time being reckoned in weeks. The temperature of the water was recorded in the morning and in the evening every day, and from these readings a monthly average was calculated. This work was first done in 1955 and was repeated during 1956 and 1957 using a fresh population of yearling fish each year.

In order to follow the growth of these fish when living wild a population of 120 was released into Scale Tarn, a small moorland tarn near Esthwaite Water, in March 1957. These fish fed naturally on the fauna of the tarn and at intervals a large sample was netted out and the lengths of the fish in the sample were measured.

Early in 1959 fifty yearling fish were released into a netted-off portion of Windermere and left there to feed naturally. Each month a large sample of these fish was netted out and the lengths of the fish were measured. Monthly growth rates were calculated as for the hatchery fish.

Results

The results of this work are shown in Figs. 1 and 2. In these graphs the monthly specific growth rates for the various populations of fish are plotted against time. It is immediately apparent from these figures that the growth rate varies in a regular annual pattern similar to the pattern of growth displayed by the older fish (Swift, 1955). The fish always show a high growth rate in the spring and nearly always in the autumn, and a low growth rate during the winter and in mid-summer.

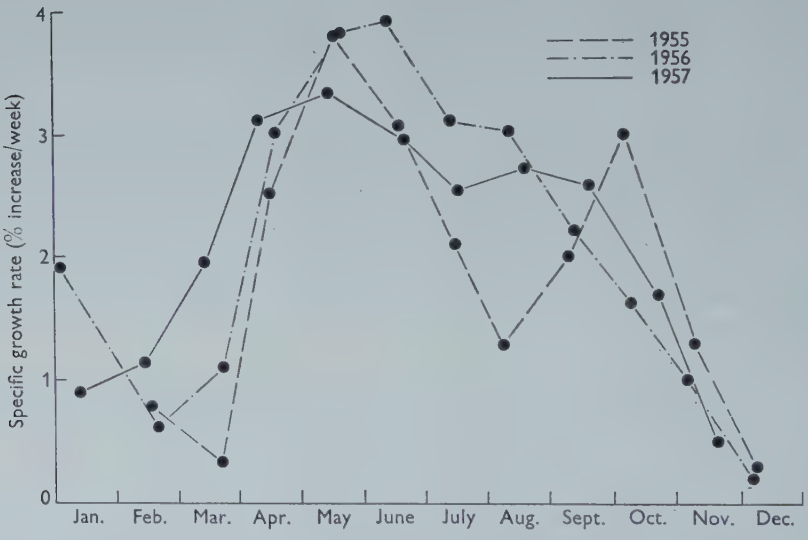

Fig. 1. The seasonal variations of specific growth rate in length of three populations of yearling trout. The growth rate is expressed as the percentage increase per week calculated from the formula

$$\frac{\ln L_2 - \ln L_1}{t_2 - t_1} \times 100.$$

The results were obtained by measuring the whole of the population.

Discussion

These results show that the growth rate of brown trout, at least in these latitudes, varies in a regular rhythmical fashion, and that this rhythm bears a definite relationship to the seasons. Furthermore, this rhythm is not affected by the state of the animals' gonads or by the type of habitat if this is not too adverse. These regular variations in growth rate must result from the response of the animal to a rhythmical change in the external environments, or from a regular endogenous rhythm in the physiology of the

animal, or from a combination of both these factors. Evidence for the existence of endogenous rhythms in animals has received renewed attention in recent years; see the reviews of Brown (1957) and Harker (1958). An endogenous rhythm in the capacity of a fish to grow could be an important factor in deciding the form of the annual growth-rate cycle in these animals. Brown (1946*b*) grew 2-year-old trout in constant-environment aquaria and obtained evidence indicating the possibility of such an endogenous rhythm in these animals. The number of fish used was small and the scatter in their growth rates during the same periods was large, making the interpretation of her results difficult. However, two interesting factors emerged from this work: first,

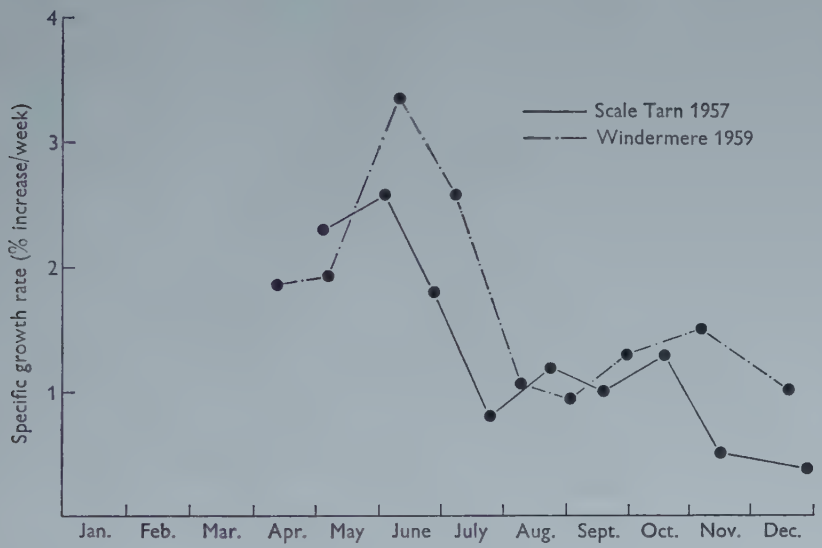

Fig. 2. The seasonal variations of the specific growth rate in length of two populations of yearling trout, one living in Windermere and the other in Scale Tarn. The growth rate is expressed as the percentage increase per week calculated from the formula

$$\frac{\ln L_2 - \ln L_1}{t_2 - t_1} \times 100.$$

her fish became ripe in the second autumn of their sojourn in the tanks, and secondly, they grew well during the winter when wild trout have a poor growth rate. It has been demonstrated that the trout, after reaching a certain physiological age, spawns as a result of the stimulus of the changing length of the daily light period (Hoover & Hubbard, 1937; Hazard & Eddy, 1951). The fact that the fish became ripe in the constant-environment tanks at the same time of the year as they would have done in the wild suggests that they already had a rhythm impressed on them since hatching.

Nevertheless, it does not seem clear that the changes in growth rate of Brown's fish were the result of an endogenous annual rhythm in the fish's capacity to grow—a rhythm in the production of growth hormone for example. Furthermore, there is little correspondence between the variations in growth rate in the tanks and the variations in the growth rate of the fish in the wild. If therefore an endogenous annual cycle in the capacity of these animals to grow does exist, it seems to have little

influence in deciding the actual growth rate of the fish in the wild. It was therefore decided to ignore the possibility of such an endogenous rhythm when considering the possible causes of annual growth-rate cycle described in this paper, and to consider the growth-rate cycle as being the direct result of an annual variation in the value of some component of the external environment. In these latitudes there are three

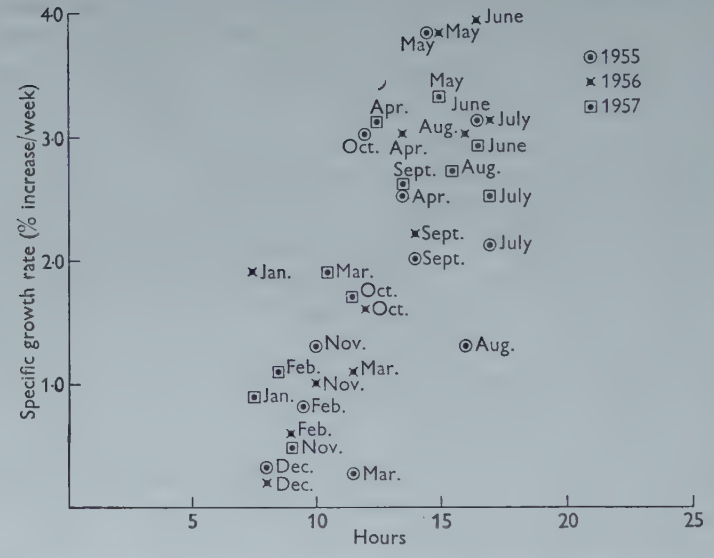

Fig. 3. The average monthly values of the specific growth rates in length plotted against the average length of the daily photoperiod over the same period of time.

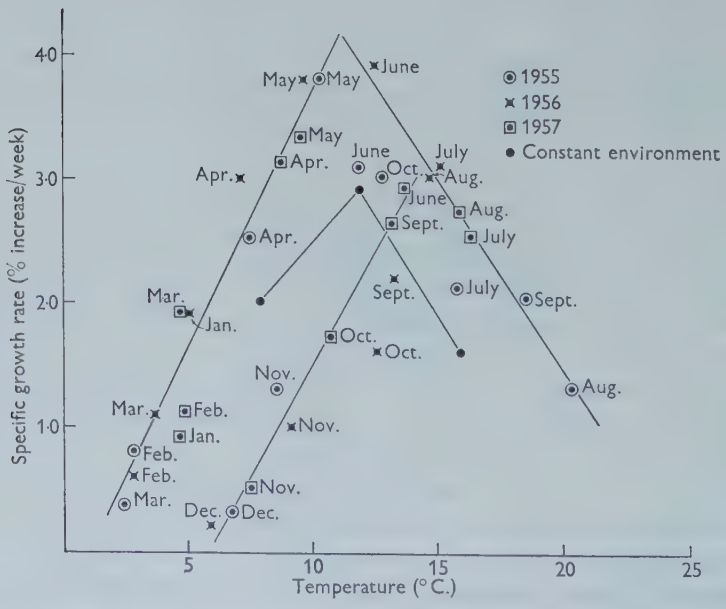

Fig. 4. The average monthly values of the specific growth rate in length plotted against the average water temperature over the same period. Also shown is the average specific growth rate in length of the fish living at different temperatures in a constant environment.

external environmental factors which vary with a frequency of 12 months: these are water temperature, photoperiod length, and concentration of solutes in the water. Changes in the solute concentration do not seem to affect the growth rate, for the order of magnitude of these changes is (for most elements) much greater in small bodies of water than in large (F. J. H. Mackereth, private communication), and it has been shown (Fig. 2) that the growth-rate cycle was the same in a large body of water (Windermere) as in a small body of water (Scale Tarn). It is a possibility that in some habitats some minor element may become limiting at some time of the year, but with our present knowledge one can do no more than note this possibility. It seems likely therefore that the growth rate is responding to change in either water temperature or photoperiod length. The average monthly values of water temperature and photoperiod length were plotted against the corresponding growth rate of the fish. There is little indication of a relationship between growth rate and photoperiod length; the slight correlation between the two (Fig. 3) is more probably the result of the contemporaneous increase in photoperiod length and in water temperature which occurs in these latitudes. Fig 4 shows the graph of growth rate plotted against water temperature. During the spring when the water temperature is rising the growth rate increases proportionally, until the water temperature reaches the 10–12° C. level; above this point the growth rate of the fish becomes inversely proportional to the temperature, so that during the summer months, as the water warms up to its maximum annual temperature and then cools, the growth rate of the fish falls and then rises. The change in the relationship between the growth rate and the water temperature which occurs between 10 and 12° C. in the spring, takes place at a higher temperature in the autumn, when the growth rate of the fish becomes proportional to the water temperature at about 14° C. This is further evidence that the photoperiod length does not, under natural conditions, influence the growth rate, for in 1955 the fall in the growth rate of the fish occurred in October whereas in 1956 and 1957 it occurred in September. The change always took place at the same water temperature but not at the same photoperiod length. If the average water temperature during the summer does not rise much above 14° C. then it follows from this relationship that the growth rate of the fish will not rise during the autumn, but will continue to fall for the rest of the year; this was the case in 1956.

PART 2. THE GROWTH RATE OF YEARLING BROWN TROUT KEPT IN A CONSTANT ENVIRONMENT

Introduction

In order to confirm the relationship between temperature and growth rate reported in the previous section a set of eight constant-environment aquaria was constructed at the Ferry House. The results of experiments in these aquaria are reported in this section.

Method

Eight light-tight aquaria, each holding approximately 100 l., were constructed of wood, lined with asphalt and insulated with granulated cork. The water supply to the tanks is pumped from Windermere through a plankton net, over an ultra-violet

sterilizer and into a header tank. Here it is cooled to 1° C. below the operating temperature of the tanks. The water enters the tank at 500 ml./min. and is stirred and aerated by compressed air entering through porous ceramic blocks. The tanks are trimmed to the operating temperature by heaters and in this way the tanks are held to within ±0·1° C. of the operating temperature. Each tank is illuminated by a 2 ft. 'daylight' fluorescent light, controlled by a mechanical time switch.

For these experiments each tank was stocked with ten yearling brown trout. The fish were individually identified by fin clips. They were fed to satiation daily with minced liver set in gelatine (Swift, 1960). Every 2 weeks they were anaesthetized with tricaine methano-sulphonate, weighed and measured.

During the course of these experiments which lasted from April to October some of the fish died; when this happened the dead fish was replaced by one from a stock tank. The stock tank was kept under the same environmental conditions as the experimental tank. Four populations of ten fish were used in this work. If a fish died during the course of the experiment its growth during the fortnight preceding its death was not used in the final assessment of its growth rate. The growth rate of a replacement fish which had not been living in the tank for more than 4 weeks was also omitted from the final calculations.

Results

All the fish were kept for 3 months at each of three temperatures, 8°, 12° and 16° C., and at each temperature were subject to 4, 8 or 12 hr. of light per day; each different photoperiod length lasted for 4 weeks thus allowing the fish to be measured twice during this time. The growth rates showed no consistent response to changes in photoperiod length. It was decided to ignore the different photoperiods used during these experiments, and to consider the change in the lengths and weights of the fish over the 12-week period for each temperature. The first and last measurements made on each fish were used to calculate the growth rate.

The histograms in Fig. 5 show the frequency distribution of the various values of the specific growth rates of the fish, when growing at the various temperatures of the experiment. The growth rate in both length and weight was larger at 12° C. than at 8° or 16° C. This supports the relationship between temperature and growth rate discussed in the previous section. The three values for the means of the specific growth rate in length obtained at the three experimental temperatures are shown on Fig. 5. These values are also plotted on Fig. 4, and it will be seen that the slopes of the lines joining these three points correspond very closely with the slopes of the lines indicating the relationship between growth rate and water temperature as deduced from the results of the experiment with the hatchery fish. These results indicate that it is the water temperature which plays the decisive role in the control of the growth rate of trout living under natural conditions, the fish achieving their maximum growth rate at 12° C.

The second point to note in these results is the very wide scatter of rates of growth of individual fish at the different temperatures of the experiment. These rates range from 0·5 to 4·0% at 8° C., from 1·5 to 4·5% at 12° C., from 0·5 to 3·0% at 16° C. It was at first suspected that this wide variation in the growth rates of individual fish at any temperature was probably the result of an order of hierarchy, such as Brown (1946*a*, *b*) demonstrated in her brown trout populations. However, an examination of

the results showed that this was in fact not the case; within a population the growth rate of the fish was found to be independent of size, some fish growing better than others for some internal rather than external reason.

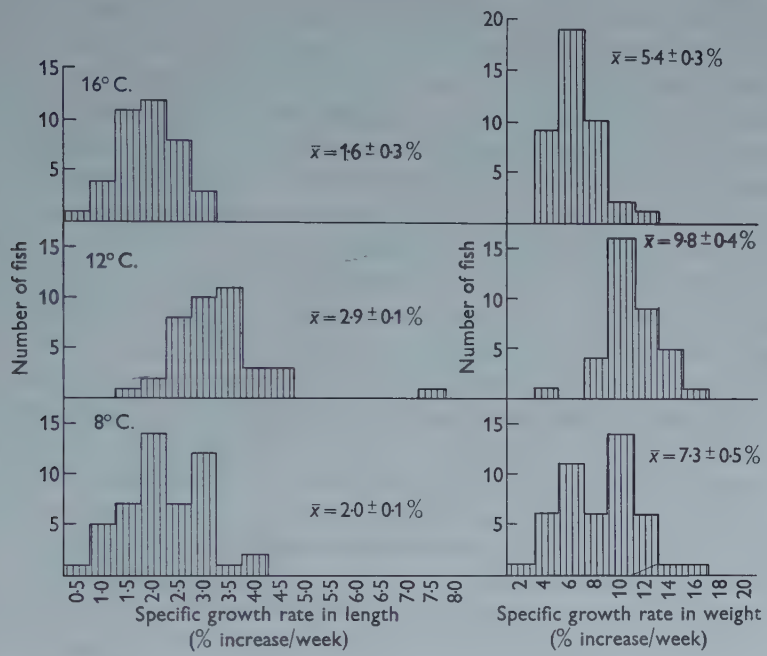

Fig. 5. The frequency distribution of the various values of specific growth rate in length and weight of the fish growing at various temperatures in constant environments. The means and the standard error of the means are shown.

Discussion

There can be no doubt that the photoperiod length does influence the physiology of fish (Hoover & Hubbard, 1937; Brown, 1946*b*; Hazard & Eddy, 1951; Hoar, 1955; Eisler, 1957).

Eisler and Brown both demonstrated a direct effect on the growth rate by the photoperiod length, but this response took some time to become apparent, 6 weeks for Eisler's fish and 2 and 5 months for Brown's; my fish showed no effect after 1 month.

It is important to try to distinguish between the effect of day-to-day changes in photoperiod length and the effect of the length of the photoperiod when this is constant from day to day. The work on the ripening of fish gonads (Hoover & Hubbard, 1937; Hazard & Eddy, 1951) has shown that for this it is the changing length of the daily photoperiod that is important. The apparent lack of any relationship between photoperiod length and growth rate of wild fish (Fig. 3) suggests that the growth rate is not affected by the natural day-to-day change in photoperiod length.

Pentelow (1939) working with brown trout found that the growth was roughly proportional to food, and that food consumption fell above 15° C., suggesting an optimum temperature above 10° C. and below 15° C. Baldwin (1956) found that *Salvelinus fontinalis* consumed most food and made their best growth at 13° C. The

optimum temperature for growth of the fish found by both these workers agrees well with the optimum temperature reported in this paper.

The only previous investigation of the growth of trout in constant environments is that of Brown (1946*b*, *c*). Brown found that her fish grew best between 7° and 9° C. and between 16° and 19° C., compared to the optimum temperature of 12° C. reported here. As has been shown in Fig. 5, at any given temperature there is a large scatter in the growth rates of individual fish. This fact coupled with the small numbers of fish used by Brown makes the interpretation of her results a little difficult, for an inspection of her Figs. 2 and 3 (Brown, 1946*c*, pp. 148, 149) shows that as the standard deviations are large it is difficult to establish with any degree of certainty the optimum temperature for growth; the high growth rate recorded at 18° C. results from the very high growth of one fish out of the population of ten. It is apparent from Table 1 that in one experiment the fish actually made their best growth at 11·5° C. This being so there is perhaps less discrepancy than might appear at first sight between Brown's results and those reported in this paper.

In a previous paper (Swift, 1955) it was postulated that 3-year-old trout living in a hatchery stewpond showed two distinct temperature optima for growth, 11° and 14° C. An examination of Fig. 3 shows clearly that these yearling trout also display maximum growth at two different temperatures. When the fish are subject to an increasing temperature their growth rate decreases above 12° C., as in the early summer of each year; when the fish are subject to a decreasing temperature, as happens in the autumn of each year, the growth rate decreases at the higher temperature of 14° C. Thus the optimum temperature for growth would seem to depend on the previous thermal history of the fish. That the thermal history of a fish affects its metabolism is well known, see for instance the reviews by Fisher (1958) and by Fry (1951). Hoar & Cottle (1952) have shown that the water and lipid content of goldfish, together with the cholesterol/fatty acid and cholestrol/phospholipid ratios, vary with the acclimatization temperature. Kanungo & Prosser (1959) found for goldfish that the oxygen consumption, measured at 20° C., was greater for fish acclimatized to 10° C. than for fish acclimatized to 30° C. and this observation is supported by the results of Fry & Hart (1948). If the water temperature can influence the physiology of the fish in this way it is perhaps not unreasonable to suggest that the previous thermal history of the fish may determine the point at which the relationship between water temperature and growth rate changes when fish are subject to a changing water temperature.

During any given period of time the growth rate of a fish must depend basically on the supply of metabolites and oxygen to the growing tissue. Ignoring food reserves in the animal, the supply of metabolites depends on the rate of digestion, and the supply of oxygen on the rate of respiration. It is possible that either or both of these two factors may decide the growth rate of the fish, and an increasing deficiency of either of them could account for a falling growth rate.

There is little evidence of the effect of temperature on the rate of digestion of fish. Maltzan (1935) working with carp, and Swift (unpublished) working with trout, found that the rate of passage of food through the gut increased with the temperature.

The respiration of fish has received the attention of many workers and the subject has recently been reviewed by Fry (1957). The oxygen content of water falls, and the oxygen requirements of a fish rise, with increasing temperature, causing the fish's

ventilation rate to rise faster than its respiration rate. The utilization of dissolved oxygen falls with increasing ventilation rates, thus greatly increasing the cost of respiration, in terms of resting metabolism, at higher ventilation rates (van Dam, 1938). These facts support the idea that the respiratory mechanism of a fish may reach the limit of its capacity at higher temperatures. Job (1955) has found that the active respiration of 45 g. *Salvelinus fontinalis* is limited by the oxygen content of water warmer than 15° C. Fry (1957) has pointed out that in fish the greatest increase in the active over the standard respiratory rate is of the order of fourfold, while in man (Krogh, 1941) the increase is 20-fold; Fry suggested that it may be the respiratory rather than the digestive system which limits the growth rate in fish. It is suggested that the fall in the growth rate demonstrated to occur in brown trout above 12° C. is the result of the increasing cost of respiration at these higher temperatures, and the increasing incapability of the fish's respiratory capacity to meet its respiratory needs. If this is so then it would seem probable that fish given a 'choice' in a temperature gradient would 'prefer' to be in a temperature near to 12° C. Fisher & Elson (1950) found that yearling trout preferred water at 10° C., at which temperature the fish made their maximum movement in response to an electrical stimulus. Brett (1952) found that young salmon of the genus (*Oncorhyncus* preferred water at 12–14° C. Garside & Tait (1958) found that *Salmo gairdneri* preferred water at 11–16° C.

It must be pointed out that there is no direct evidence to support this suggestion as to the cause of the fall in the growth rate about 12° C.; experimental evidence is needed to prove or disprove it.

SUMMARY

1. A regular annual growth-rate cycle is demonstrated in wild and hatchery yearling brown trout; the fish have a high growth rate in the spring and autumn and a low growth rate during the summer and winter of each year.

2. Experimental work with constant-environment aquaria, together with the results of the field work, indicate that the water temperature is the main external environmental factor influencing the growth rate. Maximum growth rate is achieved at 12° C.

3. The reason for the fall in growth rate above 12° C. is discussed and it is suggested that inadequacy of the respiratory system of the fish is the prime cause.

I am grateful for the assistance given to me by various members of the Freshwater Biological Association laboratory staff, in particular by Mr A. Martindale who has looked after my experimental fish. I am indebted to Mr H. C. Gilson for a critical reading of my manuscript.

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A STUDY IN INSECT MULTIPARASITISM

II. THE MECHANISM AND CONTROL OF COMPETITION FOR POSSESSION OF THE HOST

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INTRODUCTION

Many endophagous insect parasitoids require an entire host insect for their own complete development. When such a host is attacked by two species of parasitoids simultaneously it follows that a state of competition occurs between them. The occurrence of this type of multiparasitism depends primarily upon the oviposition behaviour of the two parasitoids in response to hosts that are already parasitized; the results of such multiparasitism must depend upon the outcome of interspecific competition between the immature stages of the parasitoids within the host's body.

In many cases of multiparasitism one species has an intrinsic superiority over its opponent and invariably destroys it, either by use of the mandibles (Pemberton & Willard, 1918*a*; van Steenburgh & Boyce, 1937; Ullyett, 1943; Simmonds, 1953*a*), or by an unspecified means of physiological suppression (Muesebeck, 1918; Pemberton & Willard, 1918*b*; Webber, 1932; Muesebeck & Parker, 1933; Parker, 1933; Willard & Mason, 1937; Graham, 1948; van den Bosch, Bess & Haramoto, 1951; van den Bosch, 1951*a*, *b*; Jenni, 1951).

More commonly, there is no intrinsic superiority on the part of either parasitoid, and free competition occurs between them, the victor completing its development and the loser dying, either as an egg or a young larva. Several suggestions have been made in the literature as to the possible mechanism of competition between such solitary endoparasitic species. In the first place, the older parasitoid is presumed to survive by eliminating the younger through starvation (Fiske & Thompson, 1909; Tothill, 1922), thus emphasizing the importance of time of oviposition as the determining factor in competition. Secondly, cases of direct physical attack by one parasitoid on another using the mandibles for fighting have frequently been recorded (Howard & Fiske, 1911; Thompson, 1923; Wheeler, 1923; Willard, 1927; Willard & Bissel, 1930; Bess, 1936; Eliot Hardy, 1938; Simmonds, 1944, 1953*b*). In these cases neither competitor has an intrinsic advantage over the other and the result of competition is apparently decided by the time of oviposition.

A third suggestion is that one parasite eliminates the other by physiological suppression, either by conditioning the haemolymph of the host so that it becomes unsuitable for the development of any successor (Balduf, 1926*a*, *b*; Compère & Smith,

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1927, 1932; Daniel, 1932; Cendaña, 1937; Taylor, 1937; Bess, 1939; Jenni, 1951; van den Bosch & Haramoto, 1953; Johnson, 1959), or by the postulated secretion of a toxic substance which kills the opponent (Timberlake, 1910, 1912; Spencer, 1926; Thompson & Parker, 1930).

Most of the published information on multiparasitism exists in papers that are primarily concerned with population studies on parasitoids and their use in the biological control of pests. Apart from a study by Smith (1929) the factors influencing the result of multiparasitism and the mechanism by which it is achieved have received little attention. This experimental study of the subject is divided into two parts: the behaviour of the adult female parasitoids in host selection and oviposition (Fisher, 1961) and the present paper, which is devoted to an experimental analysis of the factors which determine the result of multiparasitic competition when neither parasite has intrinsic superiority and of the exact mechanism by which the competition takes place.

MATERIAL AND METHODS

The hymenopterous parasitoids used in this study were ichneumon wasps belonging to the Ophioninae: *Nemeritis canescens* Grav. and *Horogenes chrysostictos* Gmelin. Both species attack mature larvae of the moth *Ephestia sericarium* Scott (Phycitidae), and all three species were maintained in continuous laboratory culture by methods described elsewhere (Fisher, 1961).

In the study of multiparasitism on a quantitative basis it is necessary to obtain numbers of hosts each containing one parasitoid of known age of each species. The technique of exposing a set of hosts first to *Horogenes* and then to *Nemeritis*, or of simultaneous exposure, proved to be unsuitable for quantitative work owing to erratic oviposition. Even the most favourable host–parasite ratio left some of the hosts unused by the parasites, while others received more than one egg of each species. Since anything but a 1:1 ratio between *Horogenes* and *Nemeritis* in any host would weigh heavily in favour of the superparasitizing species, measures had to be taken to ensure that there was only one egg of each species in each host used. Since preliminary experiments showed that there is no apparent interaction between the parasitoid eggs before they hatch, it was possible to attain multiparasitism of *Ephestia* larvae by the artificial injection of eggs of known age without endangering the survival of the eggs or altering the outcome of competition. The artificial injection of *Nemeritis* eggs by means of a glass micropipette has been used extensively by Salt (1955, 1956, 1957) in a series of papers devoted to the defence reactions of insects to this parasitoid. Since the eggs of *Horogenes* have a smaller diameter than those of *Nemeritis* the micropipette designed by Salt could easily be used for the simultaneous injection of eggs of both species.

Both *Horogenes* and *Nemeritis* can be induced to superparasitize *Ephestia* larvae very easily, so the problem of obtaining large numbers of their eggs raises no difficulty. For this, a standard exposure of three females to fifteen hosts for 4 hr. at 25° C. was used throughout the experimental work. For *Horogenes*, this gave a mean superparasitism of 5·12 eggs/host and for *Nemeritis*, 3·59 eggs/host. The superparasitized larvae were dissected 48 hr. later to obtain the eggs. After washing in two changes of insect Ringer, multiparasitism of a fresh series of hosts was attained by injecting

each with an egg of *Horogenes* and an egg of *Nemeritis*. The technique of injection followed that which is described by Salt (1955).

In order to reduce the risk of bacterial infection from the host cuticle each host was washed in a 0·5% solution of sodium hypochlorite before injection, and the insect Ringer, instruments and glassware were sterilized before each experiment. Following each injection the wound was sealed with a small quantity of the commercial collodion preparation 'New Skin' and the injected *Ephestia* larvae were isolated in 3 × 1 in. glass vials plugged with cotton wool and incubated at constant temperature until competition had taken place.

The results could be obtained in two ways. Either the host was left in the incubator until the victorious parasitoid emerged, or it was dissected after several days when the parasitoid larvae had reached the end of the first instar, by which time the outcome of competition had been decided. The eliminated larva could then be removed and mounted for examination. The second method was generally used, for it allowed a detailed examination to be made of the losing larva in each case of competition.

The advantage of artificial injection is that the age of the eggs can be varied at will, so as to vary the stage at which the parasitoids meet in multiparasitism. The age of each egg can be arranged so that a larva of *Horogenes*, on hatching from the egg, will meet an egg or a larva of any given age within the same host. Using this technique the effects of the relative ages of the competitors, the environmental temperature and the species of host attacked were examined individually as well as in relation to one another.

THE MECHANISM OF COMPETITION

Both *Horogenes* and *Nemeritis* readily lay their eggs in previously parasitized hosts (Fisher, 1961). Competition must therefore occur between their immature stages within a multiparasitized host, since both are solitary internal parasitoids and only one of them can develop to maturity in each host attacked.

In preliminary experiments to determine the way in which the parasite larvae competed with each other, sets of ten *Ephestia* larvae were exposed first to ovipositing females of *Horogenes* and then to those of *Nemeritis*, or to both simultaneously for a total parasitization period of 4 hr. They were then incubated for 1, 2, 4 or 6 days in separate test series before being dissected for observation of the result of competition. In each experiment only those hosts which contained a single parasite of each species were taken for examination. All superparasitized hosts were rejected.

Those examined 24 and 48 hr. after oviposition contained unhatched eggs of both species. Both were developing normally, containing embryos at the stages relevant to 24 and 48 hr. at 25° C. and there was no indication that either egg had exerted any influence over the other.

No effect was observed until the 1st-instar larvae of both species had successfully hatched from the egg. In the hosts dissected 4 and 6 days after oviposition only one parasite remained actively in possession of the host. The other parasite had been eliminated by being physically attacked by its opponent. Some of these losing larvae were of *Horogenes* and the others were of *Nemeritis*, thus demonstrating that neither species has an intrinsic superiority over the other.

In a second series of preliminary tests in which the attacks by the two parasites were separated by 3 days, the older larva was invariably found to be the survivor, and examination showed that it had won the competition by physiologically suppressing the development of the younger parasite.

Dissection of all the *Ephestia* larvae multiparasitized in the experiments, which are summarized in Tables 4–6, showed that when 1st-instar larvae of *Horogenes* and *Nemeritis* hatch from the egg at the same time, neither has a clear advantage over the other and either species may win. However, if the two are of different ages the older larva usually wins when the difference in age is less than 40 hr. at 25° C. Furthermore, if the difference in age is 50 hr. or more at this temperature the older larva always wins. This can be attributed to the fact that two mechanisms of competition occur between the same two parasite species. In the first case one larva makes a physical attack upon the other, whereas in the second it survives by physiologically suppressing the development of its opponent.

(1) *Physical attack*

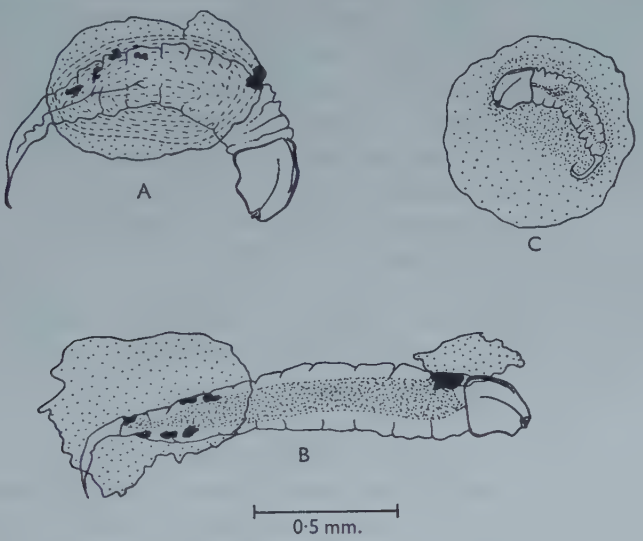
On hatching from the egg in a multiparasitized host, the young larvae of *Horogenes* and *Nemeritis* move about actively in the haemocoele of the host by vigorous movements of the abdomen and tail. When they meet, one embeds its mandibles into the body of the other and remains in this position from several minutes to half an hour (Pl. 1 *a*). After they have separated there may be some leakage of body fluid from the punctured cuticle and, in some cases, the whole of the bitten larva becomes considerably shrunken. Following this the wounded larva becomes susceptible to the blood reactions of the host, encapsulation by haemocytes and deposition of melanin at the points of wounding. Parasite larvae that have been attacked in this way show patches of melanin in one or more places on the cuticle (Pl. 1 *b*, Text-fig. 1 A) that contain pairs of distinctly darker spots. The distance between the members of each pair of spots corresponds to the distance between the mandibles of its attacker. In eliminated parasite larvae examined after 4 days at 25° C. there is an aggregation of host phagocytes around the wound, and by 6 days this enlarges considerably and either encloses the whole larva in a loose capsule or forms a tubular capsule around it (Pl. 1 *c*).

Normally one of the competitors is eliminated within 24 hr. of eclosion, but occasionally the parasites do not meet until the second day of larval life. Then the biting reaction is the same and similar host reactions of melanin deposition and phagocytosis follow, although the phagocyte capsule does not cover the whole of the now enlarged larva (Pl. 1 *d*, Text-fig. 1 B).

When the two larvae separate, the victor resumes its active life of feeding and growth while the victim ceases to feed and hardly moves at all. Melanization and encapsulation by the phagocytes of the host follow, sealing the parasite off from the internal environment of the host and probably killing it by asphyxiation (Wigglesworth, 1959*a*; Salt, 1960). From the point of view of competition the actual time of death is unimportant, for from the hundreds of multiparasited host larvae dissected it was invariably found that once a parasite had been bitten by another it had lost in competition.

(2) *Physiological suppression*

When a parasitoid attacks a host that already contains a parasitoid larva more than 50 hr. old in the 1st instar, the younger larva ceases development on hatching from the egg, does not feed and soon becomes shrunken in size. Subsequently it may be enclosed by a subspherical capsule of host phagocytes and death follows rapidly. Compact capsules of regular outline may form about *Horogenes* larvae eliminated in this way (Text-fig. 1 C, Pl. 1 *e*), whereas shrinking and 'stunting' without feeding is more typical of *Nemeritis*. In some cases the younger parasitoids become suppressed as soon as they break out of the egg shell and are discovered in dissection still half enclosed by the broken chorion and surrounded by phagocytes. In no case did the larval cuticle show the pairs of melanin spots that result from physical attack by the opponent's mandibles.



Text-fig. 1. Elimination of supernumerary larvae of *Horogenes*: by physical attack, A at 2 days, B at 4 days with melanization and partial encapsulation, C by physiological suppression with complete encapsulation.

That the competing parasitoid larvae interact through the medium of the host haemolymph was confirmed by two experiments in which physical contact between the parasites was prevented by ligaturing the body of the host with fine cotton thread. This prevented the two from making direct contact with each other, but was loose enough to allow free circulation of the host blood. The injections of eggs were so timed that the older larva was at the 1st–2nd-instar moult by the time the younger one reached eclosion. Two experiments were carried out, *Horogenes* being the younger larva in the first and *Nemeritis* the younger in the second (Table 1).

In all cases the development of the younger larva had been suppressed and each was found to be dead and partially encapsulated in the manner described. In the first experiment the ligaturing of six hosts was not successful and both larvae were recovered from the posterior half of the host. Nevertheless, the *Horogenes* had all been killed by physiological suppression and no melanized mandible marks were found on

Table 1. *Survival of competitors separated by ligature of the host at 25° C.*

Age difference between larvae	Total injected	Competition in	*Nemeritis* wins	*Horogenes* wins	Both lose	Ligature fails	Host dies
(*a*) *Nemeritis* 4 days older than *Horogenes*	18	12	12	—	—	6*	—
(*b*) *Horogenes* 4 days older than *Nemeritis*	23	20	—	19	1	—	3

* *Nemeritis* wins 6/6.

their bodies. Thus it is apparent that the means by which the older parasitoid arrests the development of the younger is carried in the blood of the host.

Several possible explanations for this kind of inhibition have already been put forward. The older parasite may secrete a substance which is toxic to others in the same host (Timberlake, 1910; Spencer, 1926), or it may have removed from the host haemolymph some substance which results in the starvation of the younger larva (Simmonds, 1943), or it may stimulate the host blood to produce more phagocytes which, though they cannot affect the older larva, are able to encapsulate and eliminate the younger ones within the same host. Another possibility is that the older parasite renders the host blood unsuitable for subsequent parasites by its own metabolic activities; that is to say, the younger larva is affected by the salivary, excretory or respiratory products of its opponent.

One of these can be ruled out without experiment. In *Horogenes* and *Nemeritis* the inhibitory factor precedes any attack by host phagocytes, for the young larva is arrested in its growth before any phagocytes begin to collect around it. Encapsulation is an effect of suppression and not its cause.

Experimental work

The first two proposed mechanisms, the secretion of a toxic substance and the removal of a growth substance, are derived from observations that younger parasites cannot survive in the same host with an older parasite but are able to do so when removed to a hitherto unparasitized host (Simmonds, 1943). To test such a possibility the converse of Simmonds's experiment of transplantation was performed—namely the observation of the effect of injecting blood from hosts containing 2nd- or 3rd-instar parasites into hosts containing eggs or very young 1st-instar larvae.

Two sets of fifteen hosts each containing a young 1st-instar larva were injected with approximately 0·0015 ml. of blood taken from hosts containing 2nd- or 3rd-instar parasites. At the same time two further sets of ten hosts were injected with blood from healthy hosts and set aside as controls. Altogether, eight of the injected hosts died after the operation, and of the surviving twenty-two, young larvae were recovered from seventeen hosts. These were alive and developing well and showed no difference in size from the control larvae.

The difficulties of transferring blood from one *Ephestia* to another are considerable and possibly the conditions of these experiments were too artificial. Avoiding these, a further experiment was carried out by injecting eggs into hosts containing killed 2nd-instar larvae. This was done by locating the 2nd-instar *Nemeritis* within its living host by strong transmitted light, manoeuvring it away from the fat body of the host

and killing it by pinching it with forceps without penetrating the host's cuticle. Single eggs of *Horogenes*, 52 hr. old, were then injected into fifteen hosts and left for 3 days before dissection. In the second replicate *Nemeritis* eggs were injected into fifteen hosts containing killed 2nd-instar *Horogenes* larvae. In both replicates all younger parasites were found alive and developing normally after 3 days.

These two experiments show first, that the haemolymph of the host had not been made permanently unsuitable by the older parasite, and second, that the arresting factor is connected with the presence of a living parasite. A theory of suppression by a toxic secretion is therefore untenable for *Horogenes* and *Nemeritis*. Yet a reversible change could be brought about as a result of the metabolic activities of the older larva.

(*a*) *Salivary secretion*

Tothill (1922) suggested that the elimination of supernumeraries of *Campoplex pilosulus* Prov. (Ichneumonidae) might be associated with salivary secretion, and Simmonds (1943) suggested that the same mechanism might occur in *Nemeritis*.

A histological examination of the paired labial (salivary) glands of *Horogenes* and *Nemeritis* showed that they are modified for silk production. Although they are well developed from the 1st larval instar onwards, they have no true salivary function but are solely concerned with production of silk which is stored in the lumen of each gland until it is used by the mature larva in spinning the cocoon.

In experimental work two sets of fifteen *Ephestia* larvae, one containing 48 hr. eggs of *Horogenes*, the other eggs of *Nemeritis*, were injected with an extract made of crushed salivary glands from 3rd-instar larvae in insect Ringer solution. No effect was observed on either group; all parasites developed normally.

(*b*) *Excretory products*

Horogenes and *Nemeritis* are among those endoparasites whose larvae have no continuous lumen between the mid and hind gut until the mature 5th-instar larva emerges from the body of its host. Serial sectioning of 1st- and 2nd-instar larvae showed that the occlusion exists at the posterior end of the sac-like mid gut. The Malpighian tubules therefore open normally into the hind gut and may be excreting through the anus, though there is reason to doubt this, since larvae of Hymenoptera are known to accumulate excretory products in urate cells of the fat body (Wigglesworth, 1953). The young larvae of these parasites also have prominent rectal glands, of unknown function, which may be associated with resorption of water and uric acid. Extracts of Malpighian tubules and of rectal glands, crushed in insect Ringer and then centrifuged, of 1st-, 2nd- and 3rd-instar parasites were injected into hosts containing eggs of the competing species. Twenty-five hosts were injected in each experiment. In some of these the parasites' development was slightly retarded in relation to the control series, but none became arrested in its growth. No pattern of inhibition and subsequent encapsulation was found in any of the experimental parasite larvae.

(*c*) *Specific inhibitor*

Since neither salivary nor excretory products appeared to have any effect on young parasitoids within *Ephestia*, if the older larva is secreting any kind of inhibiting substance, be it an ordinary product of its own metabolism or an unknown specific

inhibitor as some authors have postulated, the effect should be observable in hanging-drop cultures of *Ephestia* blood containing both young and old parasite larvae.

In an extensive series of tests, eggs of both species which were about to hatch were placed in drops of *Ephestia* blood on coverslips, inverted over deep cavity slides, and incubated at 25° C. To each, a minute drop of penicillin sulphate was added (diluted to 100 units per million) to prevent bacterial growth. The first series of eggs were placed singly in blood drawn from a host containing 1st-, 2nd- or 4th-instar parasites of the other species; in the second series the eggs were placed with live parasites of the other species in the 1st, 2nd or 4th instar. The results showed that out of sixty replicates, of all instars and blood conditions, no younger parasite became inhibited, either in blood which had previously contained a parasite, or in the actual presence of an older one, even though both lived for as long as 10 days in the same drop. The younger larvae not only hatched successfully but often developed to the 2nd instar before dying in the slowly congealing blood. These observations rule out the possibility of a specific inhibitor and salivary or excretory inhibition, all of which, were they effective, would have accumulated in the blood drop and affected the younger parasite.

Normally the younger parasite in the haemolymph of a host containing an older parasite is first inhibited in development and later attacked by the host phagocytes. In these tests where it was contained in haemolymph outside the host it is able to survive equally well with an older parasite until the drop dries up. What then are the relevant differences between the *in vivo* and *in vitro* situations? In the first instance, the activity of the haemocytes is altered in a hanging drop. After removal from the host they tend to aggregate into loose clumps within a few hours, and though remaining alive for about 48 hr., they do not attack the younger parasite and, in the few instances where they do collect round a parasite, the latter is unaffected by them. An experiment was therefore set up in which thirty young 1st-instar larvae of *Nemeritis* which had been subjected to 5 days of physiological suppression in already parasitized hosts were divided into three groups. The first set of ten were removed from them and put into hanging-drop cultures of fresh *Ephestia* blood; the second set were transferred by injection to fresh, hitherto unparasitized hosts, and the third set were left in their original hosts as controls. The first set developed normally through the 1st instar for 6 days until the drop of blood congealed. The second set regained activity and seven of them completed development and emerged as adults in 28 days, while three died in the 1st instar and were later recovered from the bodies of the emerging moths. Those left in their original hosts as controls remained suppressed and died. Clearly the host haemocytes are not responsible for the initiation of inhibition since the second set were able to complete their development and emerge as adults.

In the second instance, the hanging-drop culture method would upset any respiratory relationship between the parasite and its immediate environment, the host blood, for it would allow free exchange of oxygen and carbon dioxide between the blood and the air in the slide cavity.

(*d*) *Respiratory inhibition*

Since none of the foregoing theories of the suppression of younger parasites can be confirmed by planned experiments, and since a theory of suppression through lack of oxygen would account for the existing facts, it may well be that the older larva

utilizes all the available oxygen in the host blood, leaving the younger one retarded by partial asphyxiation, after which it is attacked by the host phagocytes. If this is so, then a decrease in the oxygen content of the host blood should suppress parasite development and an increase should lead to the survival of supernumeraries and to the removal of the suppression.

Table 2. *Summarized course of multiparasitic competition in hosts reared in a gas mixture containing* 50% O_2 *at* 25° C.

(The difference in age between the older and younger parasites is 96 hr. in both experimental and control hosts. The controls, reared in air at 25° C., show by dissection of sample hosts on days indicated by * that all younger parasites are physiologically suppressed. The younger parasites in hosts reared in 50% O_2 are not suppressed and eliminate their older competitors by physical attack.)

	Condition of parasites from hosts reared in 50% O_2		Condition of parasites from hosts reared in air (controls)	
Day	Older competitor	Younger competitor	Older competitor	Younger competitor
0	Oviposition	—	Oviposition	—
1	—	—	—	—
2	—	—	—	—
3	Eclosion	—	Eclosion	—
4	1st instar	Oviposition	1st instar	Oviposition
5	—	—	—	—
6*	1st instar	Egg, 48 hr.	1st instar	Egg, 48 hr.
7	—	Eclosion	2nd instar	Eclosion
8*	1st instar (pa)	1st instar†	3rd instar†	1st instar (ps)
9*	2nd instar (pa)	2nd instar†	4th instar†	1st instar (ps)
10*	3rd instar (pa)	1st instar†	4th instar†	1st instar (ps)
11*	4th instar (pa)	1st instar†	5th instar†	1st instar (ps)
12*	3rd instar (pa)	1st instar†	—	—
13*	4th instar (pa)	1st instar†	5th instar†	1st instar (ps)
14*	5th instar (pa)	2nd instar†	Prepupa	—
15	—	—	Pupa	—
16	—	—	—	—
17*	2nd instar (pa)	3rd instar†	Pupa	1st instar (ps)
18	—	—	—	—
19	—	—	—	—
20	—	—	—	—
21*	2nd instar (pa)	5th instar†	Adult emergence begins	None survive
22	—	—	—	—
23	—	—	—	—
24	—	—	—	—
25	—	—	Emergence ends	—
34*	None survive	Adult emergence	—	—

† Winning competitor in multiparasitism.
(pa), physically attacked larva; (ps), physiologically suppressed larva.

To test this hypothesis an apparatus was constructed which allowed known proportions of oxygen and air to be passed over parasitized larvae at a rate of 0·5 l./hr. at constant temperature and humidity. The gas mixture was equilibrated by being bubbled through a wash bottle of water inside the incubator before passing through 6 × 1 in. glass tubes containing the parasitized larvae. Gas exit from the tubes was provided by capillary valves. By this means the *Ephestia* larvae could be maintained in atmospheres of differing oxygen content throughout the period of each experiment.

A series of tests were carried out in which the two competing parasites in each host differed in age at eclosion by 3, 4, 5 and 6 days in atmospheres containing 5, 10, 20 (controls in air) and 50% oxygen.

In five experiments, each of twenty replicates, in an atmosphere of 50% oxygen no suppression of any younger larvae was observed. On the contrary, not only did the younger larvae survive, but in those tests where the difference in age at eclosion was more than 4 days, they used the well-developed mandibles of the 1st instar to attack the older larvae which had lost this armature by moulting to the 2nd instar. In other tests where the two larvae were only 3 days apart in age, some of the younger parasites were bitten by the older ones and yet remained alive and often free from haemocytes on the 7th day after eclosion. Table 2 shows a summary of the course of multiparasitism in an atmosphere containing 50% oxygen. Similarly in an atmosphere of 33% oxygen the younger larvae attacked the older ones with their mandibles and thus effectively won in competition. The development of control larvae bred singly in *Ephestia* in normal air is shown for both the younger and older competitors. It is noticeable that the rate of development of both competitors in the experimental hosts is slower than in the controls. This is apparently not due to the increased oxygen content of the atmosphere, as separate controls have shown, but, for the older parasite, due to physical attack by the younger, and for the younger, probably due to nutritional causes as the older larvae had already converted some proportion of the host into its own tissues.

Table 3. *The effect of rearing parasitoid larvae in a gas mixture containing* 50% O_2

(The retardation of development is shown by the stage of development attained on selected days as compared with control parasites from hosts reared in air. The stage of egg development is recorded as that which is normally found in eggs reared at 25° C. in air at 48 and 65 hr. after oviposition. Larval development is recorded by instar and length of the larva. (A) shows stage of development attained by a single parasite in each host; (B), from superparasitized hosts; (C), the control, shows the equivalent normal state of development of single parasites in each host reared in air.)

Age (days)	No. examined	Stage of *Nemeritis* larvae recovered (A) singly injected hosts	(B) superparasitized hosts	(C) controls reared in air
3	5	Egg, 48 hr. stage	Egg, 48 hr. stage	1st-instar larva (0·65 mm.)
4	8	Egg, 65 hr. stage	1st-instar larvae (0·7 mm.)	1st-instar larva (1·0 mm.)
7	7	1st-instar larva (1·0 mm.)	1st-instar larvae (1·0 mm.)	Mature 1st-instar larva (2·0 mm.)
8	4	1st-instar larva (1·2 mm.)	1st-instar larvae (1·1 mm.)	2nd-instar larva (2·3 mm.)

Finally, in fifteen hosts ligatured to prevent physical contact between the competing parasites, in 50% oxygen both competitors continued to develop satisfactorily for 7 days after eclosion of the younger. No younger larva became suppressed in any host.

Conversely, the effect of lowering the oxygen content of the air to 10 or 5%, by the addition of nitrogen, was to retard the development of all the parasites. The eggs did not hatch until the 4th or 5th day after oviposition at 25° C. (normal time is 59 ± 2 hr. for *Horogenes*). Many of the 1st-instar larvae did not develop and later became invested by host phagocytes. Others grew to a length of 1·0 mm., equivalent to the

normal mid-1st instar, but did not feed properly and then ceased to develop. The development of such larvae compared with those reared in air is shown in Table 3. Both in super- and multiparasitized hosts the development of all eggs and larvae was retarded and they showed all the features of physiological suppression.

THE FACTORS CONTROLLING THE RESULT OF COMPETITION

Preliminary tests of multiparasitism of *Ephestia* by *Horogenes* and *Nemeritis* showed that neither species has an intrinsic superiority over the other in this host. The phenomenon can therefore be subjected to experimental analysis to ascertain the external conditions which control its result. In this, three factors were found to be of importance: the time of oviposition by each species, environmental temperature and the species of host attacked by both parasitoids.

(1) *Time of oviposition*

Both species readily lay eggs in previously parasitized hosts (Fisher, 1961). Multiparasitism can therefore occur when a host contains two eggs, an egg and a larva, or two larvae of different species, depending upon the time of oviposition by each parasite. Since there is no mutual interaction between eggs, the experimental work is divided into two parts: competition between 1st-instar larvae, by physical combat, and between larvae in later instars and eggs or 1st-instar larvae, by physiological suppression.

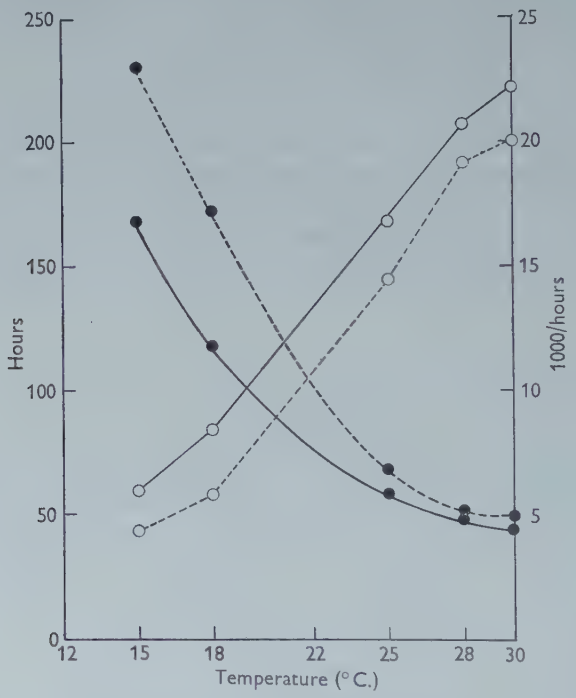
(*a*) *Competition between 1st-instar larvae*

In a preliminary test fifteen healthy mature larvae of *Ephestia* were exposed simultaneously to one female of *Horogenes* and one of *Nemeritis* for 4 hr. Six replicates were set up and incubated at 25° C. for 4 days. On dissection of all hosts, seventy-seven were found to contain larvae of both species in differing numbers, but only thirty-six of these contained a single larva of each species and could be counted for the experiment. The results showed that, although the eggs were laid at the same time (± 2 hr.), *Horogenes* defeated *Nemeritis* in twenty-four out of thirty-six cases of multiparasitism. If both species had an equal chance of surviving competition a numerical result in the ratio of 1:1 would be expected. However, *Horogenes* won on twenty-four occasions out of thirty-six, which is equal to twice the standard error of the expected ratio (S.E. $= 3{\cdot}0$, $\chi^2 = 4{\cdot}0$, $P = 0{\cdot}05$). It therefore has a significant advantage over *Nemeritis*.

Horogenes' rate of pre-imaginal development is, however, higher than that of *Nemeritis*. Its egg hatches in 59 ± 2 hr. at 25° C., whereas the egg of *Nemeritis* takes 69 ± 2 hr. (Text-fig. 2). It follows that with simultaneous oviposition the parasites are not of the same age in the 1st instar when competition takes place. If the apparent advantage possessed by *Horogenes* is due not to any intrinsic advantage other than its more rapid development, then adjustment of the time of egg laying so that both eggs hatch at the same time would make the result of competition attributable to chance. Each species would therefore be expected to win in about 50% of the competitions.

In six experiments, ninety-four hosts were injected simultaneously with one egg of *Horogenes* and one of *Nemeritis* 10 hr. older. Six days after injection the hosts were

dissected for recovery of the parasite larvae. In eight hosts, the injection had been unsuccessful through the loss of one or both eggs in the bleeding from the wounded host after injection. Sixteen hosts had died before conclusion of the experiment. Of the seventy cases of multiparasitism, in twenty both parasites were dead and encapsulated with host haemocytes. Out of the fifty remaining in which one larva had survived, *Horogenes* was successful in thirty-two and *Nemeritis* in eighteen. The number of wins by *Horogenes* was not quite equal to twice the standard error from an expected ratio of 1:1 in fifty hosts ($2 \times$ S.E. $+ 25 = 32{\cdot}072$). Contingency table and χ^2 analysis showed no significant divergence from equal survival in a 1:1 ratio. The tendency within this ratio towards survival by *Horogenes* can be attributed to the duration of the oviposition period.



Text-fig. 2. Time–temperature and temperature–rate curves for duration of the egg stage of *Horogenes* (solid line) and *Nemeritis* (broken line).

The victor of each competition was found to be active and undamaged, showing no sign of host reaction and of length varying between 1·0 and 1·3 mm., equal to the size normally attained after 6 days at 25° C. The loser had been eliminated in each case by being bitten by the victor, sometimes in several places. Melanin deposits were found at the points of wounding and each had been surrounded to a variable extent by a capsule of phagocytes of the host blood. In no case was a dead larva found without the melanin spots marking the attack by its opponent's mandibles. Their small size (0·55–0·75 mm.) indicated that fighting had taken place shortly after hatching, a conclusion which has been confirmed by the frequency with which newly hatched larvae of *Horogenes* have been dissected from their hosts still attached to their opponents by the mandibles.

To ascertain whether these results were due solely to a difference in age between the competitors in the 1st instar, a series of experiments was designed which took account of the discrepancy in duration of the egg stage between *Horogenes* and *Nemeritis* and caused one to have an excess age of 10, 20 or 30 hr. over the other in the 1st instar.

Table 4. *Results of multiparasitic competition between* Horogenes *and* Nemeritis *when the eggs of both species hatch at the same time* (± 2 *h.*), *in mature larvae of* Ephestia *at various temperatures*

Temperature (°C.)	Competition in	*Nemeritis* wins	*Horogenes* wins	Both lose	S.E. $\pm$	χ^2	*P*
15	24	11	13	—	2·45	0·16	0·2
18	23	11	12	—	2·39	0·04	0·8
25	70	18	32	20	3·54	3·38	>0·05
28	26	8	13	5	2·28	1·19	0·2

Horogenes *the older parasite in the* 1*st instar.* Four series of experiments were carried out in which the relative ages of the injected eggs were such that the *Horogenes* eggs hatched 10, 20, 30 or 40 hr. before those of *Nemeritis*. The results are shown in Table 5 and in Text-fig. 3. From these it is clear that the older *Horogenes* is, in the 1st instar, the greater is its chance of eliminating *Nemeritis* and becoming the survivor of multiparasitism. When the age difference between them is nil or only 10 hr., the result of competition is complicated by a third possibility, the death of both parasites. In such cases both larvae were found to have been bitten and by the time of dissection had both become melanin spotted and partly encapsulated. That the number of such occasions fell to zero as the age difference was increased suggests that the ability to attack, and thereby to eliminate the younger larva, increases rapidly with age in the 1st instar.

When *Horogenes* is only 10 ± 2 hr. old in the 1st instar by the time the *Nemeritis* egg hatches, this age difference is sufficient for it to survive multiparasitism in 77·7% occasions of competition ($\chi^2 = 16{\cdot}67$, $P = 0{\cdot}001$) (Table 5).

Table 5. *Results of multiparasitic competition when* Horogenes *hatches from the egg x h. before* Nemeritis *in mature* Ephestia *larvae at various temperatures*

Temperature (°C.)	*Horogenes'* excess age over *Nemeritis* at eclosion (*x*) (in hours)	Competition in	*Nemeritis* wins	*Horogenes* wins	Both lose	S.E. $\pm$	χ^2	*P*
15	35	24	6	18	—	2·45	6·0	0·01
18	40	17	3	14	—	2·062	7·12	<0·01
18	53	12	—	12	—	1·732	12·0	<0·001
25	10	58	12	42	4	3·674	16·67	<0·001
25	20	53	11	42	—	3·64	18·13	<0·001
25	30	32	6	26	—	2·828	12·5	<0·001
25	40	24	1	23	—	1·871	10·21	<0·005
28	4	16	1	14	1	1·94	11·27	<0·01
28	10	13	1	12	—	1·803	9·33	<0·005
28	20	15	1	14	—	1·94	11·27	<0·001
28	30	12	—	12	—	1·732	12·0	<0·001

When the age difference was increased in favour of *Horogenes* as the older parasite, the percentage of occasions on which it was successful rose accordingly, reaching a value of 96% when the age difference was 40 hr. When 50 or more hours older than *Nemeritis* in the 1st instar, *Horogenes* wins on all occasions of competition by physiological suppression of its opponent.

Nemeritis *the older parasite in the 1st instar*. By adjusting the times of oviposition of both parasites, series of *Nemeritis* eggs were obtained which were older than those of *Horogenes* by 10–50 hr. Four series of experiments, the results of which are presented in Table 6, show the effect of increasing age advantage of *Nemeritis* in multiparasitism. In each case the results give a highly significant divergence from the 1:1 ratio expected in equal competition. These results are in contrast with the equivalent table for *Horogenes* in that *Nemeritis* attains the 80–90% level of dominance in competition at smaller age differences. An excess age of 10 hr. over *Horogenes* gives a result of 82·5% for *Nemeritis*' success, and by 20 hr. this has risen to 95%. Nevertheless, at 25° C. the 100% level is not reached until the age difference is about 50 hr. in the 1st instar (the same as in *Horogenes*).

Table 6. *Results of multiparasitic competition when* Nemeritis *hatches from the egg x hr. before* Horogenes *in mature* Ephestia *larvae at various temperatures*

Temperature (°C.)	*Nemeritis*' excess age over *Horogenes* at eclosion (*x*) (in hours)	Competition in	*Nemeritis* wins	*Horogenes* wins	Both lose	S.E. ±	χ^2	*P*
15	30	22	16	6	—	2·345	4·545	<0·05
18	30	19	16	3	—	1·50	8·896	<0·005
25	10	40	33	3	4	3·0	25·0	<0·001
25	20	27	26	1	—	2·598	23·15	<0·001
25	30	21	20	1	—	2·29	17·19	<0·001
25	40	27	26	1	—	2·59	23·15	<0·001
28	10	27	26	1	—	2·59	23·15	<0·001
28	20	30	29	1	—	2·74	39·4	<0·001

It is therefore evident that the time of hatching from the egg is critical in deciding the outcome of competition. When one competitor is from 10 to 40 hr. older than the other, it has a statistically significant advantage but it does not have absolute superiority. However, when it has a 50 hr. advantage it wins outright in all cases of competition. Examination of the dead larvae in these latter experiments (Table 6) revealed that elimination had taken place, not by physical attack, but by physiological suppression.

(*b*) *Competition between larvae of different instars*

Observations on the oviposition behaviour of *Horogenes* and *Nemeritis* have shown that neither species completely avoids laying eggs in hosts containing advanced larval stages of the other (Fisher, 1961). The occasion may arise when a parasitoid lays an egg in an already parasitized host, and since the proportion of the pre-imaginal life spent as an egg is very small, the probability is that the second-comer will meet an advanced parasitoid larva in competition.

By using artificial parasitization the time interval between each injection was arranged so that, on hatching, the younger larva met a late 1st-, 3rd-, 4th-, or 5th-instar

larva of the opposing species. In all cases the development of the younger larva was arrested at eclosion and, if they suceeded in breaking free from the egg shell, the young larvae were rapidly enclosed by haemocytes. This result was obtained regardless of the species of the older or younger competitor.

For multiparasitism between larvae of different instars the older parasitoid invariably wins in competition, since by the time it has reached the 2nd instar it is able to suppress the survival of any subsequent parasite.

(2) *Environmental temperature*

The larval stages of an endophagous parasitoid are necessarily affected by the external physical conditions through the medium of the hosts' blood in which it lives. In this period of the life history, environmental temperature is the important factor; humidity can be disregarded when the larva lives within the body cavity of its host. Since the relative ages of the competitors are important in deciding the outcome of multiparasitism, the rate of development, regulated by environmental temperature, may be expected to affect both the result of competition and the time after hatching from the egg at which it occurs.

Table 7. *The effect of temperature on development of the eggs and 1st-instar larvae of* Horogenes *and* Nemeritis

Temperature (°C.)	*Horogenes*		*Nemeritis*	
	Time	x/time	Time	x/time
	Duration of egg stage*			
15	168 (±2) hr.	5·95	230 (±2) hr.	4·35
18	118	8·48	171	5·85
25	59	16·95	69	14·49
28	48	20·83	52	19·23
30	45	22·22	50	20·0
	Duration of egg and 1st instar†			
15	20 (days)	5·0	28 (days)	3·57
18	13	7·69	19	5·26
25	7	14·28	7	14·28
28	6	16·67	6	16·67
30	6	16·67	6	16·67

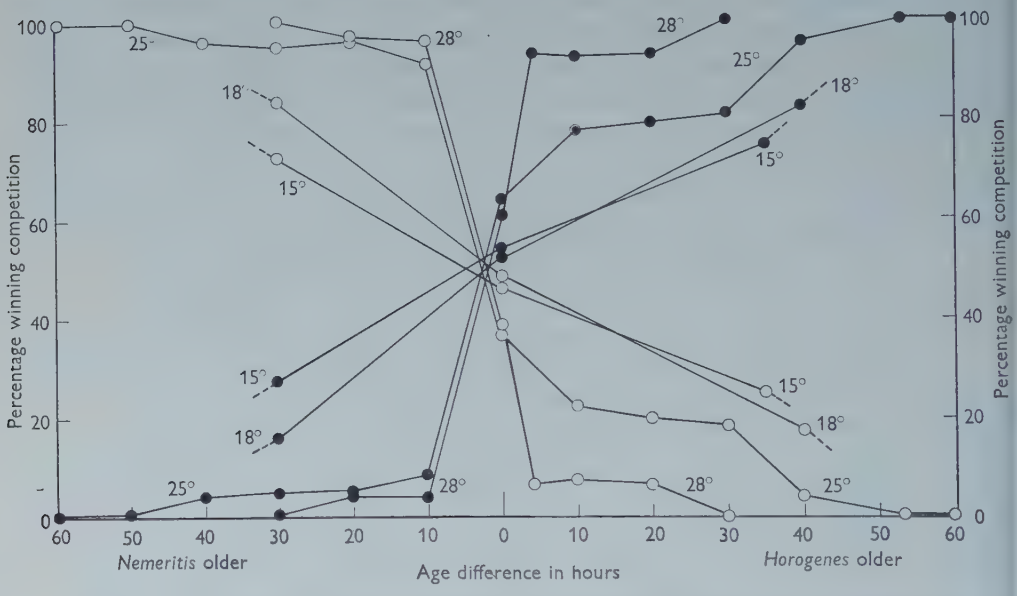
* $x = 1000$. † $x = 100$.

Observations made on the duration of the egg stage at 15°, 18°, 25°, 28° and 30° C. for both parasites showed that *Horogenes* develops more quickly than *Nemeritis* throughout this temperature range. Time–temperature and temperature–rate curves for both species are given in Text-fig. 2. The exact time (±2 hr.) for egg development at each temperature and its reciprocal are recorded in Table 7.

The results of experiments in which the two eggs hatched in the same host at the same time (±2 hr.) at 15°, 18°, 25° and 28° C. are shown in Table 4 and summarized graphically in Text-fig. 3. It may be noted that *Horogenes* wins more cases of competition than does *Nemeritis* on a simple numerical basis. A contingency table analysis for χ^2 applied to the results of Table 4 showed no significant divergence from an expected ratio of 1:1, from which it is concluded that, over the viable temperature

range of both species, neither has an intrinsic superiority over the other when the eggs of both hatch at the same time.

The combined effects of difference in age at hatching and temperature were assessed in a series of tests in which the hatching larva of each species encountered a 1st-instar larva of the opposite species from 10 to 40 hr. older than itself. The results of these, together with the χ^2 test for their significance, are given in Tables 4–6. In this way it was established that the older parasite need hatch only 10 hr. before its rival for it to become the winner of the competition. At this stage elimination takes place through direct physical attack by the older parasite, but if the older parasite hatches from the egg 50 hr. or more before its successor, it achieves absolute superiority by physiological suppression. The age difference of 50 hr. was established as the minimum



Text-fig. 3. The effect of difference in age and environmental temperature on multiparasitism between *Horogenes* (●) and *Nemeritis* (○) in the 1st instar. The results are recorded as percentages for each species on each side of the graph. The crossing point of all curves is deflected to the left of the zero age-difference line, but is within the error of each oviposition period (±2 hr.). The points on the zero line for each temperature do not differ significantly from an expected ratio of 1:1.

necessary for this effect to appear at 25° C. With the lower rate of development at 15° and 18° C. this state of dominance is not achieved until a greater age, corresponding to that reached by the 1st-instar larva at 25° C. in 50 hr. At 28° C. the rate of larval development is increased and consequently the stage of complete superiority is achieved only 30 hr. after eclosion.

The results of the combined effects of temperature and age difference in the first instar are presented together in Text-fig. 3. The outcome of competition is not fundamentally altered by environmental temperature, but the greater percentage of winnings are accorded to the older parasite at higher temperatures.

It is concluded, since at all temperatures throughout the viable range of both species *Horogenes* develops more quickly than *Nemeritis* and hatches from the egg in

advance of the latter by times varying from 4 hr. at 28° C. to 62 hr. at 15° C., that it is able to win the majority of competitions when oviposition by both occurs simultaneously.

(3) *Host species*

Smith (1929) suggested that the outcome of competition between two species in multiparasitism is influenced by the number of ecological niches which each species can occupy in any given environment. For endoparasitic species, the ecological niches can be considered to be the various species of host insects which each parasite attacks. Of the moths known to be parasitized by *Horogenes* (table I in Fisher, 1959) the following are recorded for *Nemeritis*: *Ephestia elutella* (Richards & Waloff, 1946), *E. cautella* (Chittenden, 1897; Richards & Thompson, 1932), *Galleria mellonella* (Richmond, 1925), *Achroia grisella* (Thorpe & Jones, 1937). Preliminary tests carried out on the four host species showed that both parasites will attack *E. elutella* and *E. cautella* and complete their development in them at the same rate as in *E. sericarium*. In the Galleriinae, however, the rate of development differs for the two species. Both will oviposit in *G. mellonella*, but neither will develop to maturity in it. Yet both species will successfully parasitize the small wax moth *Achroia grisella* though with markedly different rates of development. When parasitizing *Achroia* the development of *Nemeritis* is complete but very much slower than in *Ephestia*. The 1st-instar moult does not take place until the 11th day after oviposition, whereas in *Ephestia* it occurs on the 6th or 7th day. Similarly, emergence is delayed from 22–33 days in *Ephestia* to 32–33 days in *Achroia*. *Horogenes*, on the other hand, is not delayed in development and, as in *Ephestia*, emerges after 21–23 days at 25° C.

The suggestion that the rate of larval development is important in establishing the result of competition was confirmed by experiments using *Achroia* as host in which the difference in age between the larvae was altered. When there was no age difference at eclosion, *Horogenes* won twenty-three of thirty competitions (Table 8*a*). When it hatched 10 hr. before *Nemeritis*, it won twenty-nine out of thirty-one (Table 8*b*). Examination of the losing larvae from each host showed that competition took place by physiological suppression. In each case *Nemeritis* as the losing parasite had remained very small (0·6–0·65 mm.), had turned a deep dark-brown colour and was surrounded by a dense haemocyte capsule (Pl. 1*f*). The rapid establishment of physiological dominance of *Achroia* by *Horogenes* was confirmed in a third test in which its eggs hatched 34 ± 2 hr. before those of *Nemeritis* (Table 8*b*). In all hosts but one *Horogenes* had attained dominance by the time the *Nemeritis* eggs hatched, so that all the larvae of the latter died and were encapsulated soon after hatching.

To observe the effect of difference in age in *Nemeritis*' favour, eggs of *Horogenes* were injected into *Achroia* larvae at 1, 3 and 6 days after a first injection of single *Nemeritis* eggs. In the first test twenty hosts were injected with single eggs of *Nemeritis*. Twenty-four hours later they were injected with 48 hr. *Horogenes* eggs, so that the *Nemeritis* larvae were 83 hr. old when the *Horogenes* eggs hatched. Four days after the second injection all the hosts were dissected and it was found that *Horogenes*, though the younger parasite, had defeated *Nemeritis* in a significant majority of hosts by fighting (Table 8*c*).

It appears that the normal advantage of age in the 1st instar, held in this experiment by *Nemeritis*, is nullified by its slow rate of development in *Achroia*. When the time

taken by each species to complete the 1st instar is considered, it is found that, at the time of competition, *Nemeritis* has 8 days to develop to the end of the 1st instar while *Horogenes* takes only 4 days after hatching to do so. *Horogenes* can therefore be considered as being effectively 4 days older than *Nemeritis* although it hatched from the egg 24 hr. after it. This difference is sufficient to account for the dominance of *Horogenes* in this experiment.

Table 8. *Multiparasitic competition between* Horogenes *and* Nemeritis in Achroia grisella *larvae* at 25° C.

Experimental conditions	Competition in	*Nemeritis* wins	*Horogenes* wins	Both lose	S.E.±	χ^2	*P*
(*a*) *Horogenes* and *Nemeritis* eggs hatch at the same time	30	7	23	—	2·74	8·53	0·005
(*b*) *Horogenes* older than *Nemeritis* in 1st instar by 10 hr.	31	2	29	—	2·78	23·52	<0·001
Horogenes older than *Nemeritis* in 1st instar by 34 hr.	15	1	14	—	1·94	11·27	<0·01
(*c*) *Nemeritis* older than *Horogenes* in 1st instar by 24 hr.	18	2	16	—	2·12	10·88	<0·001
Nemeritis older than *Horogenes* in 1st instar by 72 hr.	14	9	5	—	1·18	1·143	0·2
Nemeritis older than *Horogenes* in 1st instar by 144 hr.	19	19	—	—	2·18	15·21	<0·001

In later experiments, when *Horogenes* was injected into *Achroia* larvae containing *Nemeritis* 3 days old, the latter achieved partial domination over the subsequent parasites (Table 8*c*), though the χ^2 test showed no particular advantage for either species in the small number of hosts injected.

However, when *Nemeritis* is injected into *Achroia* 6 days before *Horogenes* it gains absolute superiority in the host and the younger *Horogenes* larvae are physiologically suppressed (Table 8*c*).

In summarizing multiparasitism of *Achroia* it is clear that, as in the natural host, the outcome of competition is decided by the time of oviposition. However, the effect of the host on the result of multiparasitism depends upon its suitability for the growth of the competing larvae, since it is not only age, but also rate of development in the combatant instar of the parasites, which is important in determining which of two parasites shall win.

DISCUSSION

In this experimental work to ascertain the factors which determine the outcome of multiparasitic competition it has been found that the time of oviposition is of prime importance. However, since the actual competition does not occur until the eggs hatch, it follows that the result is also dependent on the rate of development in each species of host. In *Ephestia*, *Horogenes* wins in competition because its period of egg

development is shorter than that of *Nemeritis* throughout the viable temperature range of both species. This effect is emphasized in another host, *Achroia grisella*, to which they are differently adapted in respect of rate of development. Therefore, on strict application of Smith's (1929) classification of multiparasitism, *Horogenes* is intrinsically superior to *Nemeritis* when their eggs are laid at the same time. But since simultaneous oviposition is unlikely to occur in the field and the difference in the duration of the egg stage is very small, for practical purposes neither has an intrinsic superiority, and the result of competition is decided solely by the time of oviposition.

In relation to their larval environment, both species develop equally well in the lepidopterous hosts present in flour mills. Any extrinsic superiority therefore depends upon the fecundity and maximum population density attained by each. Although the fecundity of both species is approximately the same, the potential capacity for increase is very much greater for *Nemeritis* since it is a wholly parthenogenetic species. In consequence it is superior to *Horogenes* to an extent which, in field populations, far outweighs any superiority the latter may show in multiparasitism.

The elimination of supernumerary larvae of solitary insect parasitoids has been known ever since work on the biological control of insect pests began at the end of the last century. Yet, although frequently mentioned in the literature, the mechanism of competition has not been fully investigated. Using the solitary parasitoids *Horogenes* and *Nemeritis* it has been demonstrated here that competition by both physical attack and physiological suppression occurs between them. The two mechanisms occur in superparasitism and multiparasitism by both species and their appearance depends on the difference in age between the competing larvae in the 1st instar.

In the past competition has been reported to occur either by physical attack or by inhibition but not, so far as I am aware, by both reactions in any one species. The fact that they both occur in *Horogenes* and *Nemeritis* leads me to suggest that this possibility may have been overlooked by former authors, whose deductions were based on the dissection of field-collected material of unknown age. An example of this is Simmonds's (1943) statement that physical combat in *Nemeritis* is wholly insignificant in comparison with physiological suppression. Because, in his experiments, the oviposition period was not regulated, he was unable to distinguish competition between larvae of the same age from that which occurs when they are of widely different ages.

Physical attack is well known in the competition of supernumerary larvae of solitary internal parasitoids. So also is the subsequent phagocytic reaction of the host blood cells which surround the eliminated larva. Physiological suppression, however, has received very little attention, usually because it is mentioned only as a cursory observation in papers primarily concerned with the biological control of the host insect. The experimental approach taken here has shown that several of the theories by which inhibition or suppression are supposed to take place are untenable for *Horogenes* and *Nemeritis*. However, of all the possible effects of the metabolic activities of the first parasite investigated, only a gross change in the oxygen content of the host has shown a definite effect on the outcome of competition. The striking way in which the normal result of competition can be reversed by raising the oxygen in the air surrounding the host to 33 and 50% indicates that this is very probably the key to the whole mechanism of physiological suppression.

The majority of internal parasitoid larvae make no direct contact with the tracheal

system of their hosts, and rely on obtaining oxygen direct from the haemolymph in which they lie. Within the first 48 hr. of hatching from the egg the growth of the first instar larva is comparatively slow (see table II in Fisher, 1959), but thereafter growth is very rapid and the larva passes through one instar each day. It may be assumed then that the oxygen requirements of the larva increase accordingly, and on these grounds it is postulated that by the time a 1st-instar larva has lived for 50 hr. in a host at 25° C. it has reached a stage where it is utilizing all the available oxygen in its host's blood. The following hypothesis is therefore put forward: that the physiological suppression can be directly attributed to an alteration in the oxygen or carbon dioxide content of the host haemolymph, due to the increased respiratory metabolism of the oldest parasitoid larva present.

There are many references to physiological inhibition of supernumerary parasite larvae in the literature which have never been satisfactorily explained. It is suggested here that the theory of toxic secretion originally postulated by Timberlake (1910, 1912) and subsequently used by Spencer (1926) and Thompson & Parker (1930) can better be explained by lack of oxygen in the host's blood. Similarly, it can better account for suppression than the unproven theory of inhibition through starvation postulated by Tothill (1922) and later used by Taylor (1937). The hanging-drop culture experiments described here have demonstrated that, for *Horogenes* and *Nemeritis* at any rate, the younger larva does not suffer from starvation or a toxic secretion since it can develop normally and moult to the 2nd instar in the presence of an older larva. The subsequent development of suppressed larvae when they are transferred to unparasitized hosts has also shown that physiological suppression is not irreversible, and that this is strong evidence that the inhibition is metabolic and not the result of poisoning by a toxic secretion.

Many authors have observed that physiologically suppressed eggs and larvae show signs of disorganization such as stained and spotted eggs (Labeyrie, 1959), or a granulated appearance of eggs and larvae (van den Bosch & Haramoto, 1953). These have been attributed to the cytolysing action of the specific inhibitor produced by the older larva. But it is more probable that this is merely the ordinary process of cytolysis of the parasite after it has been killed by prolonged oxygen deficiency and accompanying inability to move and feed normally. Van den Bosch & Haramoto also observed that the supernumerary eggs were not killed immediately. Often the larvae hatched from the eggs before becoming suppressed, though in some cases the eggs did not hatch even when their contained embryos were fully developed. This, too, points to a metabolic inhibition rather than a specifically toxic secretion which would be expected to kill the supernumerary eggs immediately they were laid.

Oxygen-lack could also account for the suppression of gregarious larvae which has been observed when large numbers of eggs are laid in the same host (Parker, 1931; Hamilton, 1935). Supporting evidence for this theory of suppression by lack of oxygen may be gained from a number of seemingly diverse observations. Any increase in the demand made upon the host blood for oxygen would be expected to affect both younger and older parasites proportionately. Since this results in the total suppression of growth of the younger larva it will also be expected to have at least a slight retarding action on the development of the older. Compère & Smith (1927) noted that the younger chalcid larvae they were investigating were eliminated by a factor which

was also inhibiting to the survivor. Retardation of development has been noted as a feature of increasing superparasitism by Simmonds (1943) and also in the present work (cf. Table 2).

The possibility of respiratory inhibition was at least mentioned by Pemberton & Willard (1918*a*) when they wrote 'the death of the *Opius* or *Diachasma* larvae results usually from starvation, or suffocation, or possibly by the absorption of toxic excretions of the *Tetrastichus* larvae'. Compère & Smith (1932) noticed that in the elimination of supernumerary parasites of *Pseudococcus gahani* 'the phagocytic action is a secondary process acting upon organisms that have been killed by some obscure defensive host reaction'. They had observed correctly that elimination preceded phagocytosis, but failed to see that this was a result of parasitic competition and not, as they suggest, a defensive host reaction. Bess (1939) recognized that the two effects were separate, that the host reaction of phagocytosis of living parasites occurred only to those which were supernumerary, and that this phagocytosis did not occur until the eggs were ready to hatch. Both of these examples separate the actual suppression from attack by host phagocytes and the latter rightly associates them with supernumerary parasitism in these hosts which do not normally show reactions to single parasitoids of these species.

A recent observation by Lewis (1960) of a correlation between the difference in age of gregarious parasitoids in multiparasitism of spruce budworm larvae can easily be explained by respiratory suppression. He found that the longer the time interval between first attack by *Apanteles fumiferanae* and the second attack by *Glypta fumiferanae*, the greater was the percentage of *Glypta* which became suppressed. On the theory advanced here, the older the *Apanteles* larvae were, the greater their oxygen requirements became, and consequently an increased percentage of the younger *Glypta* larvae became suppressed.

There have been other suggestions of respiratory inhibition in parasites which may have some bearing on this explanation of physiological suppression. Keilin (1944) observed that dipterous parasites are considerably modified by their need for oxygen and stated that they do not grow or feed actively until contact with air is established. It has also been suggested that parasitoid larvae surrounded by phagocyte capsules in immune hosts are ultimately killed by asphyxiation (Muldrew, 1953; Wigglesworth, 1959*a*; Salt, 1960). There have also been indications that extremely localized sensitivity to oxygen lack may occur in tracheoles (Thorpe, 1936; Locke, 1958) and in epidermal cells (Wigglesworth, 1959*b*).

Clearly the sensitivity to oxygen-lack in fluid environments is important at the cellular and supra-cellular levels and there is sufficient evidence presented here to suppose that deficiencies in this respect, due to the metabolism of a well-developed parasite, are sufficient to suppress the development of supernumerary parasite larvae in the same host.

SUMMARY

1. In multiparasitism of larvae of the moth *Ephestia serecarium* by the solitary ichneumonid endoparasitoids *Horogenes chrysostictos* and *Nemeritis canescens*, neither has an intrinsic superiority over the other and free competition occurs between their immature stages for possession of the host.

2. There is no interaction between the eggs of these species before they hatch.

3. When larvae of these two species are present in the same host at the same time they compete for possession of the host by one of two mechanisms, physical attack or physiological suppression.

4. When the larvae hatch from the eggs at the same time, neither has a clear advantage over the other and either species may win by physically attacking the other with its mandibles.

5. If they are of different ages, the older larva usually wins by physical attack when the difference in age is less than 40 hr. at 25° C.

6. If the difference in age is 50 hr. or more at 25° C. the older larva always wins in competition by physiological suppression of the younger one.

7. The factors which are important in determining the result of competition are: difference in age between the 1st-instar larvae at eclosion, environmental temperature and the species of host attacked, which determine the parasites' rate of development.

8. A hypothesis of asphyxiation is presented with experimental evidence of its validity as an explanation of the physiological suppression of supernumerary parasitoids. It is suggested that asphyxiation by oxygen-lack can better account for physiological suppression than the theories of toxic secretion, specific inhibitor and starvation.

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EXPLANATION OF PLATE

(*a*) Competition by physical attack in which one *Horogenes* larva embeds its mandibles into the cuticle of the other (× 60).

(*b*) First-instar *Nemeritis* larva eliminated by physical attack and showing melanin at points of wounding and partial encapsulation (× 60).

(*c*) First-instar *Horogenes* larva eliminated by physical attack, showing melanization and a tubular haemocyte capsule (× 40).

(*d*) First-instar *Nemeritis* larva, 6 days old, melanized but only partly encapsulated after physical attack 48 hr. after eclosion (× 30).

(*e*) Physiologically suppressed 1st-instar *Horogenes* larva 3 days after eclosion (× 50).

(*f*) First-instar *Nemeritis* larva physiologically suppressed by *Horogenes* in *Achroia grisella* (× 80).


a
b
c
d
e
f

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THE MOVEMENTS OF SODIUM IONS IN THE ISOLATED ABDOMINAL NERVE CORD OF THE COCKROACH, *PERIPLANETA AMERICANA*

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INTRODUCTION

In an earlier investigation it was demonstrated that the exchange of sodium and potassium ions between the haemolymph and the central nervous system of *Periplaneta* occurred relatively rapidly (Treherne, 1961). On the basis of these results it was concluded that a dynamic steady state rather than a static impermeability must exist across the perilemma in this insect. The experiments described in this paper represent an attempt to throw some further light on the nature of the ionic movements between the haemolymph and the central nervous system in the cockroach.

METHODS

The abdominal nerve cords used in these experiments were loaded with radiosodium by the injection into the haemolymph of 100 μl. of a solution containing ^{24}Na (0·1–0·5 mc./ml.). After 30 min. the abdominal nerve cord was quickly removed from the insect, by dissection from the dorsal surface, and cleaned of adhering tissue by lightly drawing it across a piece of filter paper. In a limited number of experiments the nerve cords were loaded *in vitro* by soaking in an oxygenated solution containing ^{24}Na. The abdominal nerve cord was ligatured at the connective anterior to the first abdominal ganglion and immediately in front of the terminal abdominal ganglion. This preparation was then quickly washed in Ringer solution, tied to a length of glass rod and placed in the apparatus used to measure the sodium efflux of the abdominal nerve cord.

The apparatus used in these experiments consisted of a small Perspex trough, the floor of which was formed by a layer of 0·00025 in. terylene sheet of minimal stopping power to the β and γ radiations emitted by ^{24}Na. Immediately beneath the terylene window was a GM tube (Mullard MX 123) which was linked to a scalar unit (Panax 100c). Oxygenated Ringer flowed through the trough at a rate of approximately 50 ml./min. and the decline in radioactivity associated with the nerve cord was followed throughout the experiment. The volume of the fluid contained in the Perspex trough was 0·45 ml. The whole of the apparatus was housed in a lead castle to minimize the effects of extraneous radiations. This apparatus is essentially similar in principle to that used by Hodgkin & Keynes (1955) to study the loss of sodium ions from isolated squid giant axons.

The solution used for loading the nerve cords with ^{24}Na was that devised by Treherne (1961) and is summarized in the first column of Table 1. The relatively high concentrations of trehalose and glutamine used in this solution made it too expensive for use with the large volumes necessary in the efflux experiments. A second solution was, therefore, used in these experiments in which the trehalose was replaced by sucrose and the glutamine by an increase in the concentration of glucose (column 2, Table 1). Sodium-free solutions were prepared by replacing the sodium salts with choline chloride (column 3, Table 1) or by an appropriate concentration of xylose (column 4). In the remaining solution used in these experiments the potassium was replaced by a proportional increase in the NaCl content (column 5).

Table 1

Substance	Normal Ringer (mM./l.)	Efflux Ringer (mM./l.)	Na-free Ringer (mM./l.)	Na-free Ringer (mM./l.)	K-free Ringer (mM./l.)
NaCl	154·8	154·8	—	—	167·1
KCl	12·3	12·3	12·3	12·3	—
$CaCl_2$	4·5	4·5	4·5	4·5	4·5
$MgCl_2$	4·0	4·0	4·0	4·0	4·0
$NaHCO_3$	2·1	2·1	—	—	2·1
NaH_2PO_4	0·1	0·1	—	—	0·1
$KHCO_3$	—	—	2·1	2·1	—
KH_2PO_4	—	—	0·1	0·1	—
Trehalose	36·9	—	—	—	—
Sucrose	—	36·9	36·9	36·9	36·9
Xylose	—	—	—	281·2	—
Glucose	2·2	35·0	35·0	35·0	35·0
Glutamic acid	35·0	35·0	35·0	35·0	35·0
Glutamine	30·0	—	—	—	—
Glycine	30·0	30·0	30·0	30·0	30·0
Choline chloride	—	—	157·0	—	—

RESULTS

The decline in radioactivity of a ^{24}Na-loaded isolated abdominal nerve cord maintained in flowing Ringer solution is illustrated in Fig. 1. In all of these experiments there was an initial approximately exponential decline in radioactivity which eventually appeared to give way to a second slower phase which also approximated to an exponential curve. Unfortunately the second phase occurred in a region of very low activity and could, for example, have resulted from some errors in the estimation of the background count, although the fact that it was present in all of the experiments would make this seem rather unlikely.

It seemed possible that some of the loss of ^{24}Na from the isolated nerve cord could have occurred from the cut ends of the segmental nerves. This possibility was eliminated in some experiments by following the decline in radioactivity associated with single ligatured abdominal connectives. The connectives used were those from between the fourth and fifth abdominal ganglia. The efflux of ^{24}Na from this preparation appeared to be essentially similar to that from the whole abdominal nerve cord (Fig. 2).

When the nerve cord was loaded with ^{24}Na *in vitro*, by soaking it in radioactive Ringer solution, the initial rapid phase was significantly reduced. Fig. 3 illustrates

the rate of loss of radiosodium from a nerve cord loaded in isolation for 10 min. where the apparent onset of the slow phase occurred after less than 5 min. and at a relatively high level of radioactivity within the nerve cord. In this case then the slow exponential phase cannot be attributed to errors in the estimation of the background activity.

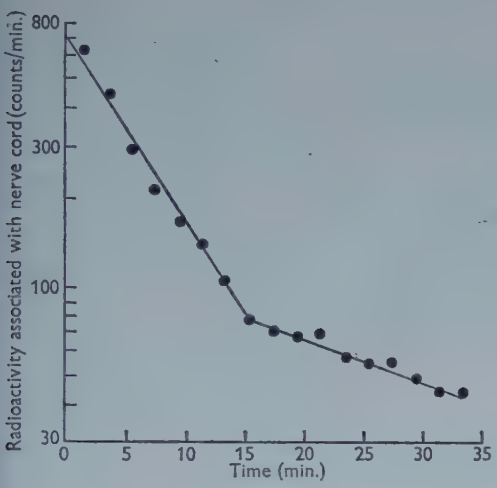

Fig. 1

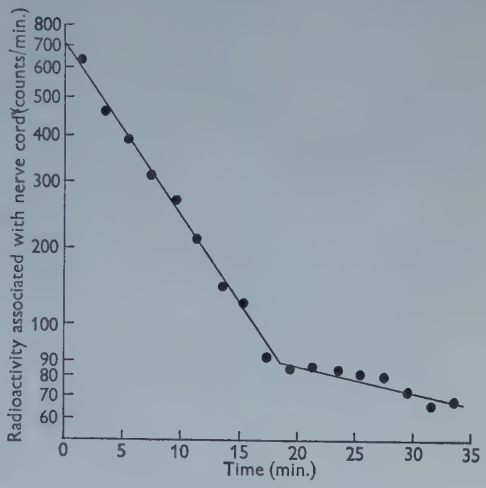

Fig. 2

Fig. 1. The efflux of ^{24}Na from an isolated abdominal nerve cord.

Fig. 2. The efflux of ^{24}Na from an isolated abdominal connective.

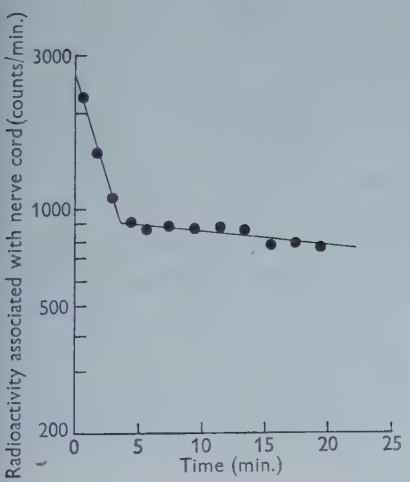

Fig. 3

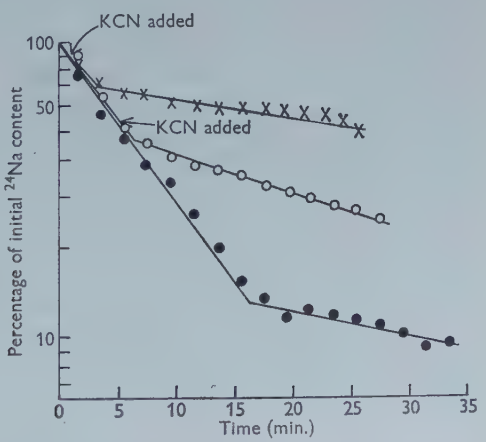

Fig. 4

Fig. 3. The efflux of ^{24}Na from an isolated nerve cord which was loaded *in vitro* for 10 min.

Fig. 4. The effect of 10·0 mM./l. KCN on sodium efflux.

The action of potassium cyanide on the rate of loss of sodium ions is shown in Fig. 4. In these experiments the normal solution was changed, after varying periods, to one containing in addition 10·0 mM./l. KCN. The addition of the poison resulted after a short delay period in a slowing down of the efflux of sodium ions.

The action of 2:4-dinitrophenol at a concentration of 0·5 mM./l. was essentially similar to that of cyanide and resulted in every case in a clear-cut reduction in the rate of loss of sodium ions from the abdominal nerve cord (Fig. 5).

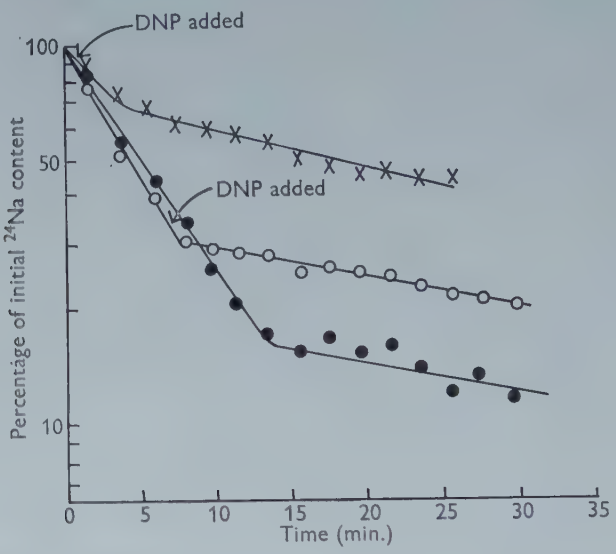

Fig. 5. The effect of 0·5 mM./l. 2:4 dinitrophenol on sodium efflux.

In the next group of experiments the effect of the external sodium concentration on the rate of loss of radiosodium ions from the abdominal nerve cord was investigated. Fig. 6 shows the decline in radioactivity of an abdominal nerve cord in which a sodium-free solution was substituted for the normal Ringer after 8 min. This solution, in which the sodium was replaced by choline, produced no marked effect on the rate of loss of sodium ions from the nerve cord. Similarly in experiments in which the sodium was replaced by an appropriate concentration of xylose there was very little effect on the initial exponential efflux from the nerve cord (Fig. 7).

The rate of loss of sodium from the nerve cord in the potassium-free solution is illustrated in Fig. 8. In this case there was slowing down in the rate of loss of sodium ions in the potassium-free medium. On return to the normal solution the efflux appeared to continue at the rapid rate before the onset of the final slow phase of sodium loss from this system. There was some variation in extent of the effect of the potassium-free solution on sodium loss. This variation can be seen in the results summarized in Table 2.

DISCUSSION

The initial exponential efflux of radiosodium from the abdominal nerve cord has been shown to be significantly reduced by cyanide and dinitrophenol which clearly suggests that the exit of sodium ions measured in these experiments is not a passive process, but is linked in some way with the metabolism of the system. This inhibitory effect is consistent with the results which have been obtained with several other cells and tissues including the squid axon (Hodgkin & Keynes, 1955). Dinitrophenol, at the order of concentration used in these experiments, is generally held to interfere

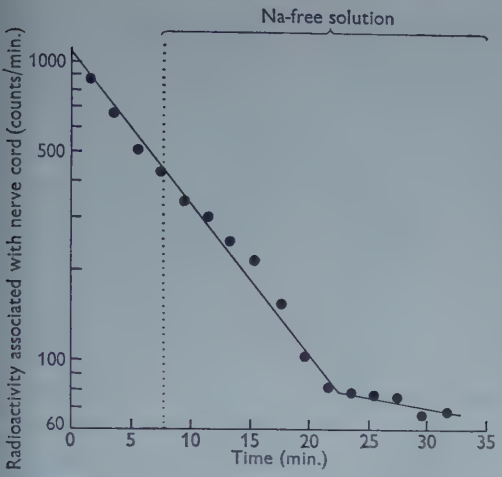

Fig. 6

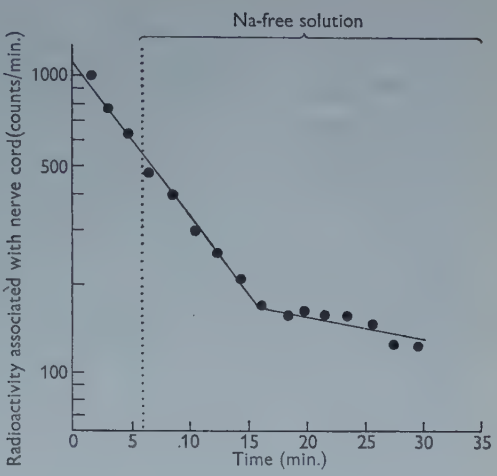

Fig. 7

Fig. 6. The efflux of sodium in a normal solution and in one in which the sodium was replaced by choline.

Fig. 7. The efflux of ^{24}Na in a normal solution and in one in which the sodium was replaced by xylose.

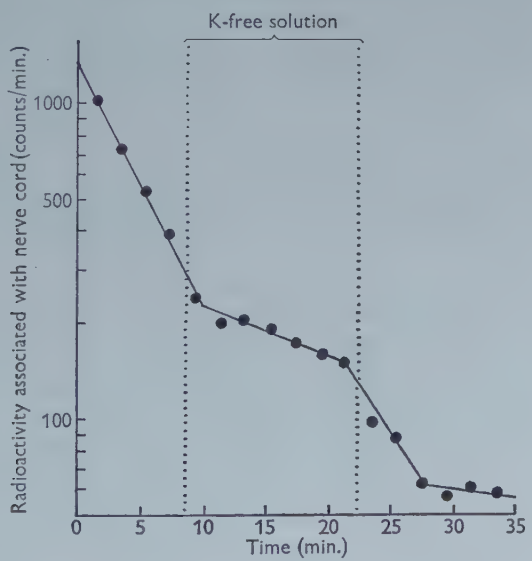

Fig. 8. The effect of potassium-free solution on ^{24}Na efflux from the isolated abdominal nerve cord.

with oxidative phosphorylation and in fact a dependence of sodium extrusion on the presence of ATP has been recently demonstrated in the squid axon (Caldwell, Hodgkin, Keynes & Shaw, 1960).

The extrusion of sodium from the isolated nerve cord appeared to approximate to a two-stage process—an initial rapid exponential phase eventually giving way to a slower component. The second phase was unfortunately difficult to establish in these experiments as it occurred in a region of very low activity, although its presence can perhaps

Table 2. *The effect of potassium-free solution on the rate of sodium efflux ($t_{0.5}$) from isolated nerve cords*

Serial	$t_{0.5}$ Normal solution (min.)	$t_{0.5}$ K-free solution (min.)
1	5·5	19·4
2	5·2	11·8
3	4·8	11·6
4	5·0	35·0
5	5·4	12·6
6	4·9	12·2
7	6·0	25·2

be inferred from the observations on nerve cords loaded *in vitro* in which a slow phase was demonstrated at a high level of radioactivity. There is, of course, no *a priori* reason for assuming that sodium efflux from this organ should be a simple exponential process. It might be imagined, for example, that such an effect could result from the presence of rapid and slowly exchanging fractions within the nerve cord, although it might not perhaps be expected that the phase change would occur so abruptly. It is, however, very difficult to visualize how in such a system a short *in vitro* loading with ^{24}Na could result in an apparent increase in the slowly exchanging sodium fraction. The similarity of the second phase of sodium efflux to that obtained in the poisoned preparations (Figs. 4, 5) might suggest that this slow phase does in fact represent some sort of breakdown of the normal extrusion mechanism in the isolated nerve cord. This evidence is of course entirely circumstantial, but it is perhaps significant to recall the observation of Hoyle (1953, p. 123) that the nerve cord of *Locusta* only showed normal electrical activity for relatively short periods when isolated and separated from its tracheal supply.

The rate of loss of ^{24}Na from the isolated nerve cord was not appreciably reduced when the external sodium was replaced by choline or xylose. Thus, as was suggested in an earlier paper (Treherne, 1961), the extrusion of sodium ions from the central nervous system of this insect does not appear to be part of any 'exchange diffusion' mechanism. The fact that the rate of loss of sodium was, however, reduced in the potassium-free solution demonstrates a close relation between sodium efflux and potassium influx. Such a coupling of sodium and potassium movements has been demonstrated in several cells and tissues (cf. Hodgkin, 1958) and has led to the hypothesis that it might be the result of a mechanism by which one sodium ion is extruded for each potassium ion absorbed (Harris, 1954; Hodgkin & Keynes, 1954). In the present experiments the rate of sodium efflux in the potassium-free solution did not fall to the same extent as that in the presence of the metabolic inhibitors. In this case, as with the *Sepia* axon (Hodgkin, 1958), it is very difficult to be sure that the concentration of potassium ions immediately surrounding the nerve cord surface had not been raised by a leakage of potassium which might allow some limited coupled exchanges of sodium and potassium to continue. It is, therefore, not possible to postulate whether the coupling between sodium efflux and potassium influx is a rigid a partial one.

It is now relevant to consider the significance of these observations on sodium efflux in relation to some previous conclusions on the nature of the permeability processes associated with the continuous fibrous and cellular membrane, the perilemma, surrounding the central nervous system in this insect. The work of Twarog & Roeder (1956), following that of Hoyle (1953) on peripheral nerve, showed that in solutions of high potassium concentration the blocking-time was reduced when the nerve cord was partially desheathed. On the basis of these observations it was suggested that the perilemma functioned as a diffusion barrier restricting the movements of sodium and potassium ions and acetylcholine molecules between the haemolymph and the central nervous system. Some more recent work has, however, demonstrated rapid influxes of sugar molecules and of potassium and sodium ions into the abdominal nerve cord of *Periplaneta* (Treherne, 1960, 1961). It was concluded from these results that in fact a dynamic steady state rather than a static impermeability must exist across the perilemma in this insect (Treherne, 1961). This present investigation has shown in addition that this steady state is at least partially effected by a metabolically maintained linked-sodium pump. It seems reasonable to assume that these exchanges between the haemolymph and the central nervous system are regulated by the perineurium or some deeper cellular layer, the overlying fibrous neural lamella being probably freely permeable to ions and molecules (Wigglesworth, 1960).

In some future investigations an attempt will be made to identify more precisely the rate-limiting processes involved in the extrusion of sodium ions from the central nervous system in this insect.

SUMMARY

1. The rate of loss of sodium ions from the abdominal nerve cord of *Periplaneta* has been determined by following the decline in radioactivity of ^{24}Na-loaded nerve cords isolated in flowing Ringer solution.

2. In all of the experiments there was an initial rapid exponential decline in radioactivity which eventually gave way to a second slower phase.

3. The initial exponential extrusion of sodium ions was appreciably reduced by the presence of potassium cyanide and 2:4-dinitrophenol.

4. The rate of sodium efflux was not reduced in sodium-free solutions, but was decreased in the absence of external potassium ions.

5. It is concluded that sodium ions are extruded from the nerve cord by a metabolically maintained secretory mechanism which is also associated with the uptake of potassium ions.

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THE RELATIONSHIPS BETWEEN NUTRITION, HORMONES AND REPRODUCTION IN THE BLOWFLY *CALLIPHORA ERYTHROCEPHALA* (MEIG.)

II. THE EFFECT OF REMOVING THE OVARIES, THE CORPUS ALLATUM AND THE MEDIAN NEUROSECRETORY CELLS UPON SELECTIVE FEEDING, AND THE DEMONSTRATION OF THE CORPUS ALLATUM CYCLE

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INTRODUCTION

Calliphora females select 'protein' (Marmite in milk) and carbohydrate (sugar in water) solutions in proportions which vary with the different phases of the reproductive cycle (Strangways-Dixon, 1959, 1961). Since non-reproducing females (fed on sugar solution but no 'protein') show no signs of cyclical feeding, it seemed possible that selective feeding was a response to the cyclical requirements of the developing ovaries. Since ovarian development in *Calliphora* is controlled by the corpus allatum (c.a.) and the median neurosecretory cells (m.n.c.) of the brain (E. Thomsen, 1940, 1942, 1948, 1952) it seemed possible that selective feeding was also influenced by these secretory organs. The effects of extirpating the ovaries, the m.n.c. and the c.a. upon selective feeding were therefore investigated.

METHODS

The three operations—ovariectomy, allatectomy and removal of the m.n.c.—were performed as described by E. Thomsen (1942, 1952) with the following modifications. Loops of fuse wire instead of plasticene strips were used to hold the insects in position and (in allatectomy and m.n.c. removal) to prise the heads away from their bodies. During ovariectomy operations, the positions of the ovaries varied slightly, making it difficult to be certain that the probing forceps had grappled the ovaries rather than the convoluted gut. In the latter case, the gut was often torn and the fly had to be discarded. Therefore, instead of grappling directly for the ovaries, use was made of the rich tracheal supply which derives from the spiracles situated on the fourth and fifth abdominal segments. A small tear was made just posterior to the spiracle on the fifth abdominal segment, the forceps were inserted through the opening and grappled the tracheae as they emerged from the spiracle. The tracheae were pulled out 'hand over hand' using two pairs of forceps until the ovaries themselves emerged from the slit. The gonads were held with one pair of forceps whilst the tracheae and oviduct were cut with the other pair. The cut ends were pushed back into the abdomen and the wound was sealed with 60° C. wax. The operation was then repeated for the other ovary. It is important to perform this operation as soon after emergence

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as possible because after the flies have fed, the chances of perforating the gut increase.

The selective feeding technique and the method for culturing the flies has been described in a previous paper (Strangways-Dixon, 1961).

THE EFFECT OF OVARIECTOMY UPON SELECTIVE FEEDING AND CORPORA ALLATA VOLUMES

Selective feeding

Nineteen ovariectomy and thirty-two control operations were performed on the day of emergence. After 6 days of sugar diet [females are unable to reproduce on a sugar diet (Fraenkel, 1940), so this initial 6 days in a sugar culture was to give the flies time to utilize their remaining pupal protein reserves and thus remove a variable factor which might have influenced selective feeding] fifteen ovariectomized and eight operated control females were isolated in feeding units. Ten ovariectomized and five operated control flies were given carbohydrate and 'protein' solutions whilst the remainder were given carbohydrate solution only. Selective feeding continued for 15 days during which all carbohydrate controls and three 'protein'-ingesting (ovariectomized) females died. The latter are excluded from the graph but the former are included up to the day before death. After the experiment, the flies were dissected and the c.a. and the ovaries were examined. The 'protein'-ingesting controls contained mature ovaries. The averaged ingestions of the ovariectomized females are shown in Fig. 1. Since the selective feeding of the operated controls was cyclical, averaged ingestions would conceal the individual peaks and so are avoided. For this reason the feeding of only a single female is shown (Fig. 2). In this female, as in the others, the characteristic carbohydrate cycle is still apparent.

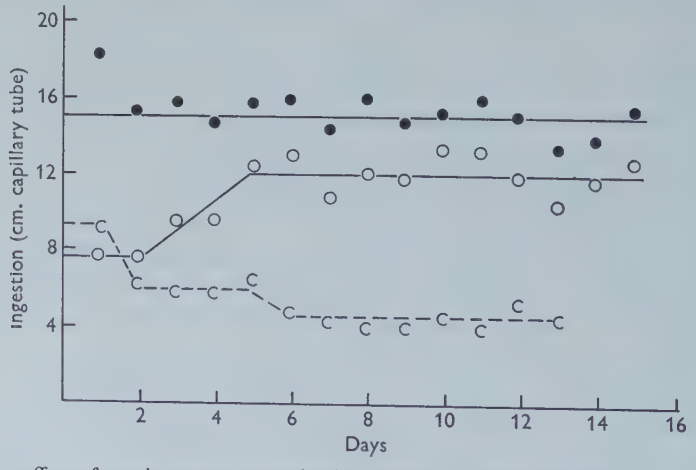

Fig. 1. The effect of ovariectomy upon selective feeding. ●, total ingestion. ○, carbohydrate ingestion. C, carbohydrate ingestion of control 'sugar' flies.

Fig. 1. shows that the carbohydrate ingestion of an ovariectomized fly—provided the fly is also allowed 'protein'—increases from an initial low level but then remains high. 'Protein' intake, on the other hand, is high initially but decreases to a constantly low level, and the total ingestion remains the same throughout. This selective feeding

is the same as that of a normal reproducing female (Strangways-Dixon, 1961, Fig. 8) except that the cycle is not repeated. It is therefore concluded that *selective feeding is independent of direct ovarian control*, but that *the ovaries—probably because they utilize the ingested foods—are responsible for the succession of one cycle upon the other.*

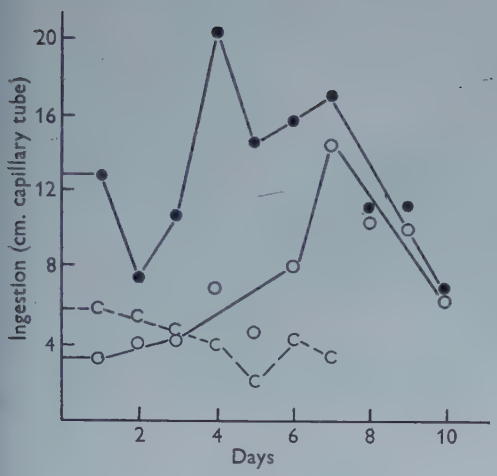

Fig. 2

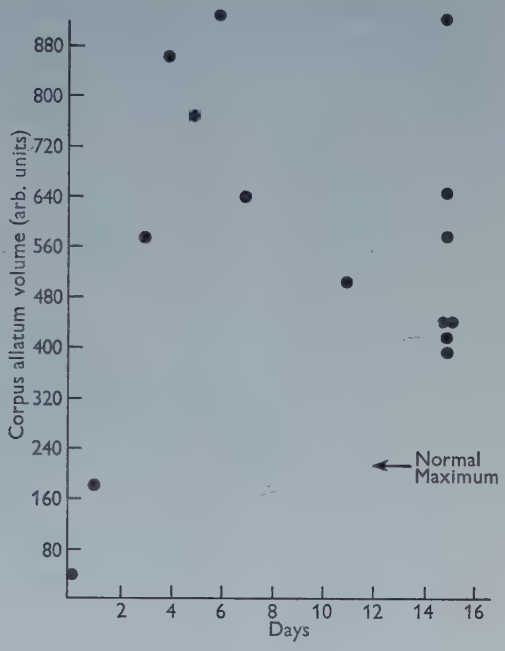

Fig. 3

Fig. 2. Operated controls for ovariectomized females. As for Fig. 1.

Fig. 3. Corpora allata volumes of ovariectomized females.

Corpora allata volumes

A second experiment was conducted synchronously with the one described above and its purpose was to follow the volume changes of the c.a. of ovariectomized females.

Of the twenty-four females which were castrated on the day after emergence, sixteen died during the experiment. The unusually high mortality was probably due to the forceps piercing the alimentary canals which were swollen after 24 hr. feeding. The flies were maintained as a sugar culture for 6 days and then meat was added. The females were dissected during the succeeding 11 days and the c.a. volumes were plotted against the number of days after the addition of meat to the diet (Fig. 3). The points shown on the fifteenth day are those of the seven females which survived the selective feeding experiment described in the preceding section. It can be seen that the c.a. of ovariectomized flies become hypertrophied by the third day and remain thus for at least 15 days. This confirms E. Thomsen's results (1940, 1942).

THE EFFECT OF ALLATECTOMY UPON SELECTIVE FEEDING

On the day of emergence, twenty-one females were placed in feeding units and were fed carbohydrate solution only. On the following day, twelve were allatectomized and six control operations were performed. The remaining three females were left as

unoperated sugar controls. The flies were starved for 24 hr. and were then allowed to ingest carbohydrate solution for a further 4 days. At this stage, 'protein' solution was added to the diet of all operated flies and selective feeding began.

At the completion of 14 days selective feeding, eight of the allatectomized females still survived. Four of these contained mature ovaries but in the others, egg development had stopped at the yolk deposition stage. The selective ingestions of the latter were averaged and the results are shown in Fig. 4.

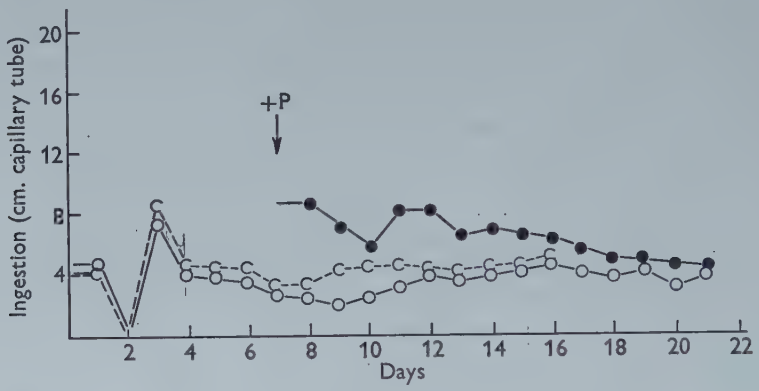

Fig. 4. The effect of allatectomy upon selective feeding. +P, 'protein' was added to the diet at this point. Otherwise as for Fig. 1.

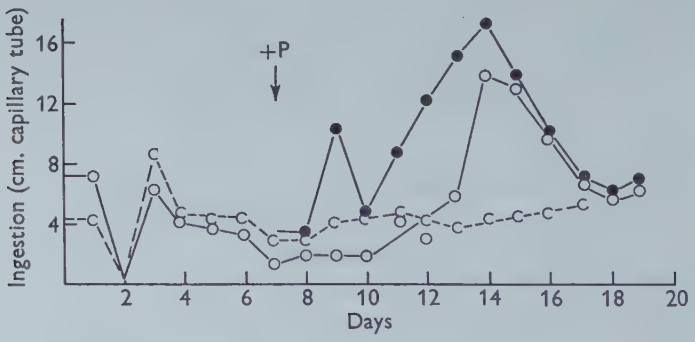

Fig. 5. Operated controls for allatectomized females. +P, 'protein' was added to the diet at this point. Otherwise as for Fig. 1.

The five surviving operated controls all contained mature ovaries. The selective feeding of one female and the averaged ingestions of the sugar controls are shown in Fig. 5. The carbohydrate ingestion of the operated control shows the normal cycle whilst the sugar control flies, as usual, consume a constantly low volume of carbohydrate.

Fig. 4. indicates that allatectomy results in carbohydrate ingestion remaining constantly low in spite of the fact that considerable quantities of 'protein' are ingested. It is therefore concluded that *the increase in carbohydrate ingestion* (which normally occurs during yolk deposition) *is dependent upon the presence and activity of the* c.a. (see Strangways-Dixon, 1959).

THE EFFECT OF OVARIECTOMY COMBINED WITH ALLATECTOMY UPON SELECTIVE FEEDING

Ovariectomy results in the increase of carbohydrate consumption to a constantly high level (Fig. 1). Allatectomy results in carbohydrate consumption remaining constantly low (Fig. 4). If, as was thought, the normal increase in carbohydrate ingestion was dependent upon the c.a. and was independent of the ovaries, then the extirpation of both these organs should result in a selective feeding similar to that of an allatectomized female.

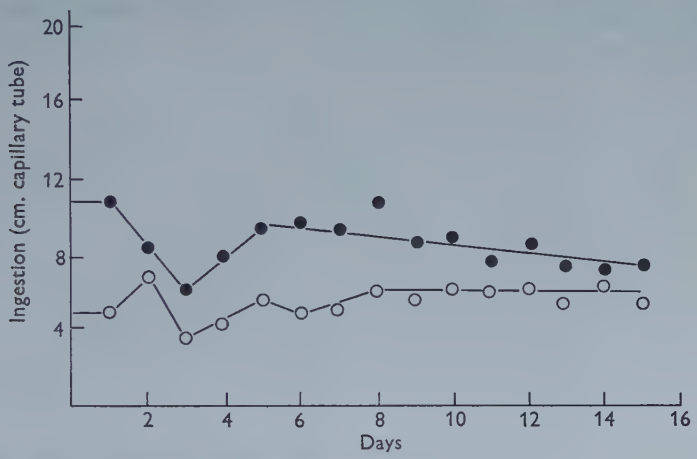

Fig. 6. The effect of ovariectomy together with allatectomy upon selective feeding. As for Fig. 1.

Twenty-four females were ovariectomized on the day of emergence and, after 6 days in a sugar culture, sixteen of the twenty-three survivors were allatectomized. This experiment was run in conjunction with those mentioned earlier in this paper and so control allatectomy operations were performed on twelve of the ovariectomy controls not in use in the other experiments. After a further 3 days in a sugar culture, the flies were isolated in feeding units. Twelve ovariectomized-allatectomized females and eight operated controls survived up to this period. Eight of the former and five of the latter were fed carbohydrate and 'protein' solutions and the remainder received carbohydrate solution only. All carbohydrate controls and one fly from the mixed diet groups died early in the experiment and so are excluded from the graphs. The selective ingestions of the double-operated females are averaged (Fig. 6), but since the ingestions of the controls were cyclical, only a single representative example is shown (Fig. 7.)

It is concluded from these results that ovariectomy combined with allatectomy results in carbohydrate consumption remaining at a constantly low level (Fig. 6). This supports the hypothesis that c.a. activity induces an increase in carbohydrate consumption.

THE CORPUS ALLATUM IN RELATION TO AGE, SIZE OF FLY AND REPRODUCTION

Since it was thought that c.a. activity induced an increase in carbohydrate ingestion, it followed that the cyclical ingestions of reproducing females should be a result of cyclical fluctuations in the activity of the c.a. The c.a. of reproducing females were therefore examined in the hope of discovering cyclical changes in volume.

A total of 105 females were removed from a reproducing culture, normally in daily batches of five, over a period of 27 days from emergence. These were dissected and their c.a. and ovaries were measured in arbitrary units (see Strangways-Dixon, 1961). Flies caught whilst ovipositing were noted.

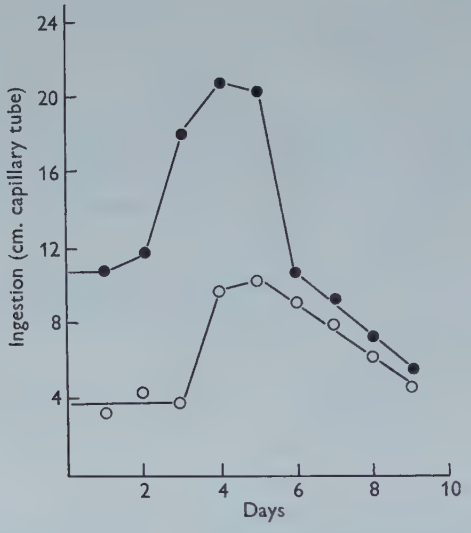

Fig. 7. Operated controls for ovariectomized allatectomized females. As for Fig. 1.

The relation of the c.a. volumes to age is shown in Fig. 8. The results confirm E. Thomsen's (1942) results in that the c.a. of mature females are larger than those of newly emerged flies. An additional observation is that the c.a. volumes taken from different flies of the same age on any single day show a very large scattering which does not seem to alter with age. Fig. 9 indicates that the C.A. volumes are not related to the lengths of the females as the scatter of the c.a. volumes is similar for each of the three lengths of fly dissected. Since there was so much variation in c.a. volumes at any given age, it was considered possible that more accurate information would be obtained if these volumes were plotted against the lengths of eggs taken from the same fly. In flies with mature eggs, however, c.a. volumes are plotted against the secondary oocytes (Fig. 10). The latter comparison is necessary because reproductive cycles tend to overlap and so the sequence of events is lost in a gravid female unless the succeeding cycle is taken into consideration.

Fig. 10 shows that the volumes of c.a. in reproducing females fluctuate cyclically. *The* c.a. *increase in size during the early stages of egg development and decrease in volume during yolk deposition* (see Strangways-Dixon, 1959). This supports the hypothesis that c.a. activity influences carbohydrate consumption.

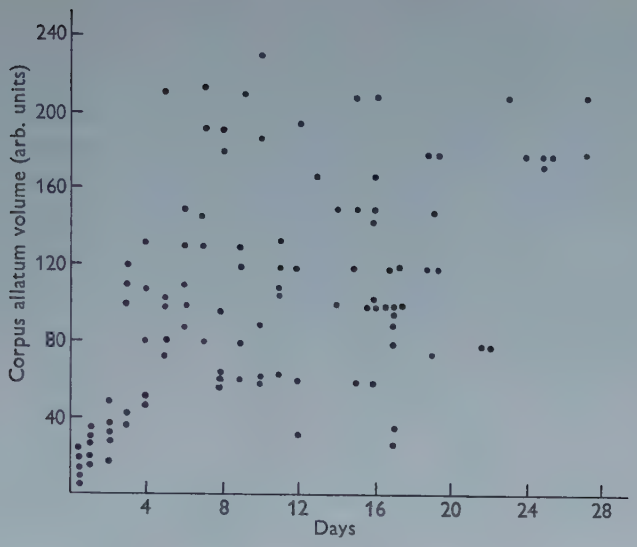

Fig. 8. Corpora allata volumes in relation to age.

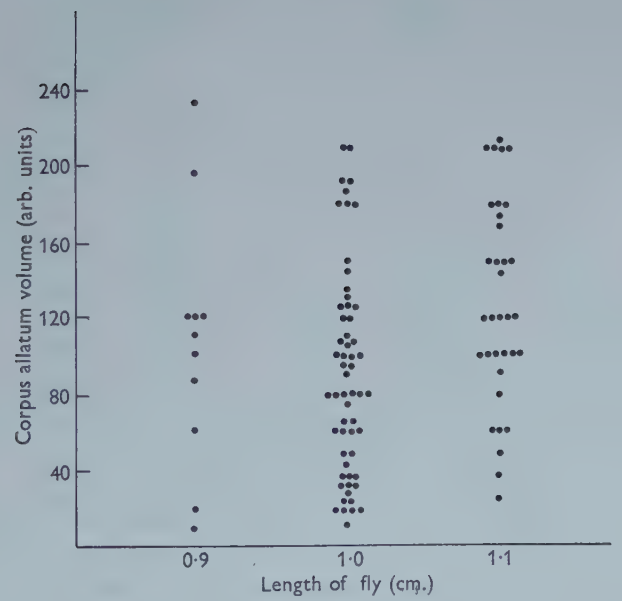

Fig. 9. Corpora allata volumes in relation to the lengths of flies dissected.

THE EFFECT OF REMOVING THE MEDIAN NEUROSECRETORY CELLS UPON SELECTIVE FEEDING

Having investigated the influences of the ovaries and the c.a. upon selective feeding, attention was turned to a third possible controlling factor—the m.n.c. The extirpation of these cells is known to prevent egg development in *Calliphora* (E. Thomsen, 1948, 1952).

The m.n.c. were removed from forty females and control operations were performed on thirty females within 24 hr. of emergence. After 6 days of sugar diet the thirty

surviving females (lacking m.n.c.) and sixteen of the operated controls were isolated in feeding units. Twenty-four of the former and ten of the latter flies were given carbohydrate and 'protein' solutions whilst the remainder received carbohydrate solution only. Thirteen mixed diet and four carbohydrate females (lacking m.n.c.), and two mixed diet and four carbohydrate females (operated controls) died during the remainder of the experiment. The ingestions of flies without m.n.c. are averaged and the carbohydrate controls are included up to the day before death (Fig. 11). For the usual reason, only a single control fly is represented (Fig. 12) and since carbohydrate ingestion is cyclical, it is assumed that the operation without removing the m.n.c. does not appreciably influence selective feeding.

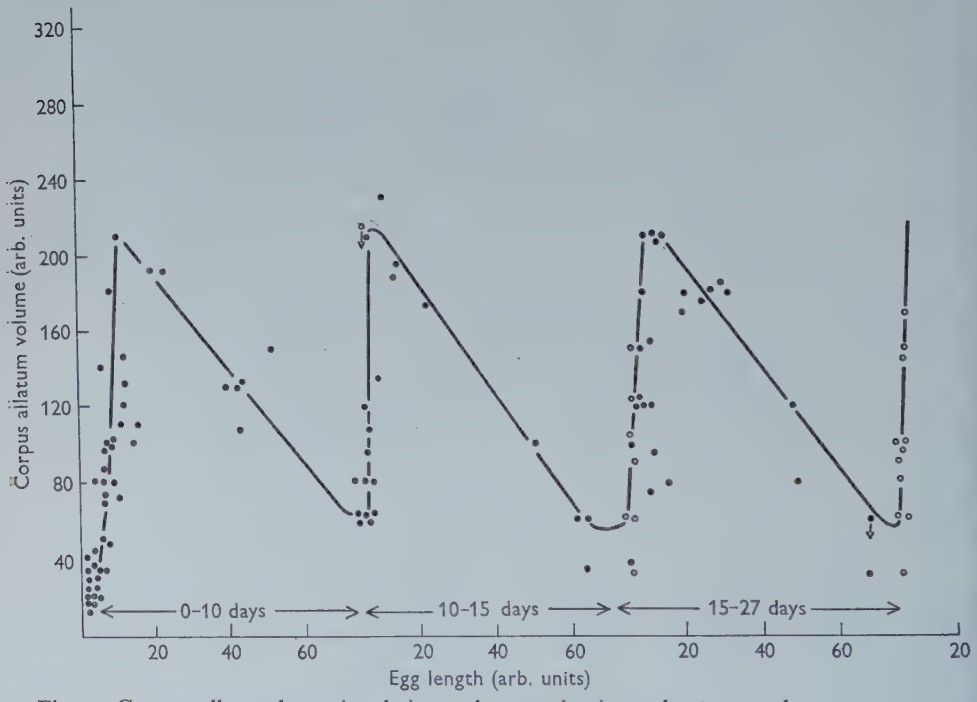

Fig. 10. Corpora allata volumes in relation to the reproductive cycle. ●, normal measurements. ○, oocytes adjacent to mature eggs. ↓, these flies were ovipositing when caught for dissection.

A total of two sugar controls and seven 'protein'-ingesting flies (lacking m.n.c.) were removed from the units during the experiment. These were dissected and the c.a. were removed and measured. The volumes of the c.a. were smaller than those of reproducing females and were only slightly larger than those of sugar flies. These results confirm those of E. Thomsen (1952).

Fig. 11 indicates that flies lacking m.n.c. ingest a constantly low volume of carbohydrate. 'Protein' ingestion is high on the first day only and then declines abruptly to negligible quantities. It appears that the *removal of the* m.n.c. *results in the failure of females to ingest 'protein' except for an initial brief period.* The preliminary intake of 'protein' may be a consequence of some residual neurosecretion in the blood or in the corpus cardiacum. It is as well to mention at this stage that at the time of this

experiment (April 1958) E. Thomsen was performing similar investigations (E. Thomsen & Møller, 1959) in which she found that females lacking m.n.c. continued to ingest meat normally. We have discussed this apparent contradiction and can find no answer except that possibly the attraction of meat is so strong that it overcomes the effect shown in the present experiment in which a solution of milk and Marmite was used instead of meat. Conclusions arising from the results where this contradiction is applicable must therefore be regarded as only very tentative.

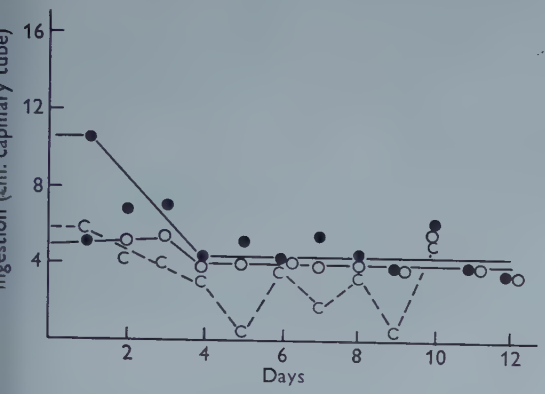

Fig. 11

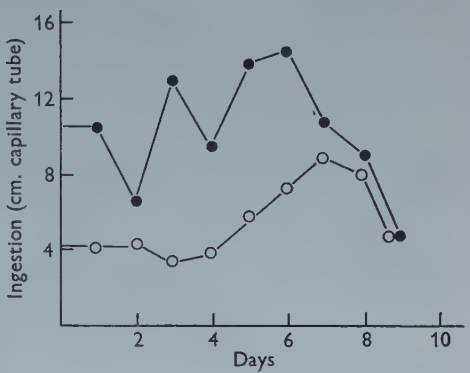

Fig. 12

Fig. 11. The effect of removing the median neurosecretory cells upon selective feeding. As for Fig. 1.

Fig. 12. Operated controls for removal of the median neurosecretory cells. As for Fig. 1.

It became apparent towards the end of the experiment that if females (without m.n.c.) did not ingest 'protein', then any previous results obtained by the same operation were in fact due to the influence of the m.n.c. upon the ingestion of 'protein'. It was decided therefore to *force* the flies, which remained at the end of the experiment, to ingest 'protein' and to see whether the ovaries and the c.a. would then develop. Of the four remaining females, two were fed on carbohydrate solution and the other two on a mixture (1:1) of carbohydrate and 'protein'. The two latter flies ingested the mixture as if it were carbohydrate and were thus forced to consume the 'protein'. On dissection—after 6 days of this treatment—'protein' could be seen in the guts of the insects, so there was no doubt about its having been ingested, and yet neither the ovaries nor the c.a. had increased in size; they were the same size as those of the carbohydrate controls. This suggested that the 'protein' had not been made available to the organs in question and that possibly the m.n.c. are necessary for the digestion of 'protein' materials. These results support the much stronger evidence of E. Thomsen & Møller (1959) who showed that the production of protease was dependent upon the presence of m.n.c. The fact that the c.a. remained small supports the hypothesis (E. Thomsen, 1952; M. Thomsen, 1954) that the presence of the m.n.c. is necessary for c.a. activity. In the next paper, evidence will be submitted suggesting that this influence is indirect (due to the influence of the m.n.c. upon digestion) since, in the normal fly, c.a. volume varies in proportion to the amount of 'protein' ingested.

These results and conclusions will be discussed together with additional relevant data in a later paper.

SUMMARY

1. A study has been made of the effects of removal of ovaries, copora allata (c.a.) and median neurosecretory cells (m.n.c.) upon the selection by female blowflies of carbohydrate (sugar in water) or 'protein' (Marmite in milk).

2. Extirpation of the ovaries resulted in high carbohydrate–low protein selection and in hypertrophy of the c.a.

3. Extirpation of the c.a. resulted in low carbohydrate selection.

4. Extirpation of both ovaries and c.a. resulted in low carbohydrate selection.

5. These and other results suggest that selection is independent of direct ovarian control, but that the ovaries influence selection in that they utilize the ingested foods and thus bring about the succession of feeding cycles.

6. The c.a., whose volume (activity?) changes cyclically during each cycle of reproduction, appears to control the fluctuations in carbohydrate consumption.

7. The m.n.c. seem to be necessary for the ingestion of 'protein' and for the activity of the c.a.

8. Reproductive cycles tend to overlap. The succeeding cycle in a gravid female must be taken into consideration when events are being related to reproduction.

Thanks are due to Prof. V. B. Wigglesworth, F.R.S., for encouragement, advice and facilities, and to the Colonial Office which supported this investigation with a research grant.

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THE URINE OF *GAMMARUS DUEBENI* AND *G. PULEX*

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(*Received* 24 *April* 1961)

INTRODUCTION

The amphipod, *Gammarus duebeni*, is essentially a brackish-water species though it extends its range into fresh water in a number of places. (Kinne, 1959; Hynes, 1954.) It occurs in fresh water apparently only when *G. pulex* is absent (Hynes, 1954) and in brackish water also competition with *G. salinus* and *G. zaddachi* appears to limit its range (Kinne, 1959). In consequence the distribution of *G. duebeni* is discontinuous and it is most commonly found in physiologically 'difficult' habitats where its competitors are absent. Such habitats include brackish-water lagoons, splash-zone rock pools, ditches near the sea and salt marsh pans (references in Kinne, 1959). Since such habitats are all subject to extensive and rapid changes in salinity as a result of inundation by sea water or fresh water, *G. duebeni* might be expected to have a high degree of osmoregulatory capacity.

Beadle & Cragg (1940) have shown that *G. duebeni* tolerates salinites in the range 100 to 2% sea water and can withstand direct transference between these two extremes of concentration. The osmotic pressure of the blood is maintained at a higher level in 2% sea water than is that of the fresh-water species *G. pulex*. In more saline media the blood concentration of *G. duebeni* rises slowly until it becomes effectively isotonic with the medium when the latter is more concentrated than about 50–60% sea water. The mechanisms responsible for the maintenance of the relatively constant blood composition when the medium is less concentrated than 50% sea water have not been fully investigated. Beadle & Cragg (1940) have shown that *G. duebeni* loses salt at a rapid rate when the animal is placed in distilled water. Rapid changes in the blood concentration may therefore be expected to follow changes in the concentration of the medium. In such circumstances any mechanism tending to retain ions within the body would clearly be of benefit by serving to slow down the change in blood concentration. One such process might be the production of urine hypotonic to the blood.

Schwabe (1933) demonstrated that the fresh-water *G. pulex* has a longer excretory tubule in the antennary gland than has the brackish and marine species *G. locusta*. By analogy with the situation in the decapod Crustacea it has been assumed that this indicates that the fresh-water species can produce hypotonic urine. Recent measurements by Hynes (1954) have extended Schwabe's work by showing that in *G. duebeni* the eosinophil section of the excretory tubule is almost twice as long as that in *G. locusta* of similar size and some three-quarters the length of the corresponding section in *G. pulex*. Hynes concludes that *G. duebeni* might also be capable of producing

hypotonic urine. This observation is of particular interest as no brackish-water crustaceans have previously been thought to produce hypotonic urine. Beadle (1943) regards the capacity to produce hypotonic urine as being one of the later physiological modifications to be developed by fresh-water animals. Further, Potts (1954) has concluded that the production of hypotonic urine by a semi-permeable animal in brackish water would contribute little towards decreasing the expenditure of energy on osmoregulation, though having a marked effect if the medium were fresh water.

In the present paper it will be shown that *G. duebeni* is not only capable of producing hypotonic urine when in brackish water or fresh water, but that it also has the capacity to vary the urine concentration as the blood concentration changes.

MATERIALS AND METHODS

Materials

G. duebeni was collected from the salt-marsh at Aberlady (Firth of Forth) and from the Stour estuary at Flatford Mill. *G. pulex* was obtained from the Braid Burn, Edinburgh. In the laboratory stocks of *G. duebeni* have been maintained in their natural medium and fed on 'Bemax' and *Enteromorpha*. They reproduced and appeared normal in every way. Species were identified by reference to Segerstråle (1959) and Reid (1944).

Methods

Analysis

Osmotic pressure determinations on both blood and urine were made using the cryoscopic method of Ramsay & Brown (1955). Osmotic pressure is expressed in terms of the concentration of NaCl (in mM./l.) having the same freezing-point depression. Tritium was counted using a standard liquid phosphor (Popop, Naphthalene and 2,5-diphenyl oxazole) in a Panax liquid scintillation counter. ^{22}Na was counted by a standard Geiger–Muller tube and Labgear Dekatron scaler. At least 2500 counts were taken on each sample reducing the expected error of the count to $\pm 2\%$.

Urine collection

A small hole was burnt with a heated needle through a 1 in. square of thin rubber sheet and the animal was inserted so that it was firmly gripped in the region between the second and third coxal plates. The rear portion of the body, with the gills, thus lay on one side of the membrane and the head and excretory apertures on the other. The membrane was placed over the mouth of a tube containing 50 ml. of the experimental medium, so that the animal's gills were immersed and allowed room to beat. The entire tube was then immersed about $\frac{1}{2}$ in. below the surface of liquid paraffin. The head and that portion of the thorax exposed to paraffin were carefully dried with filter paper. When the excretory papillae are quite dry urine collects as discrete droplets on the top of each cone.* Such droplets were sucked directly into the pipettes used for freezing-point determinations. Samples were only taken when such a droplet had been observed to form on the excretory papilla so there was no possibility that urine samples were contaminated by extraneous water collecting round the mouth parts.

* This method for the collection of urine from gammarus was first devised and used by Mr T. D. Iles.

Blood samples

Blood samples were obtained by snipping off the terminal segments of the flagellum of the first antenna. Successive sampling was possible if only a few segments at a time were taken. The blood was collected into freezing-point pipettes as it emerged and the osmotic pressure was determined at once.

RESULTS

When *G. duebeni* is acclimatized to media more concentrated than 50% sea water, the urine is isotonic with the blood. In media less concentrated than 50% sea water hypotonic urine is formed. The lower the concentration of the medium the greater is the concentration difference between blood and urine. These results are summarized in Fig. 1. The values shown are all for animals acclimatized for 3 days or longer to the experimental medium. As the concentration of the urine frequently rises when the animals have been in the collecting vessel for some time, only the lowest value recorded for each animal is given. Not infrequently the two urinary papillae produce urine of different concentrations.

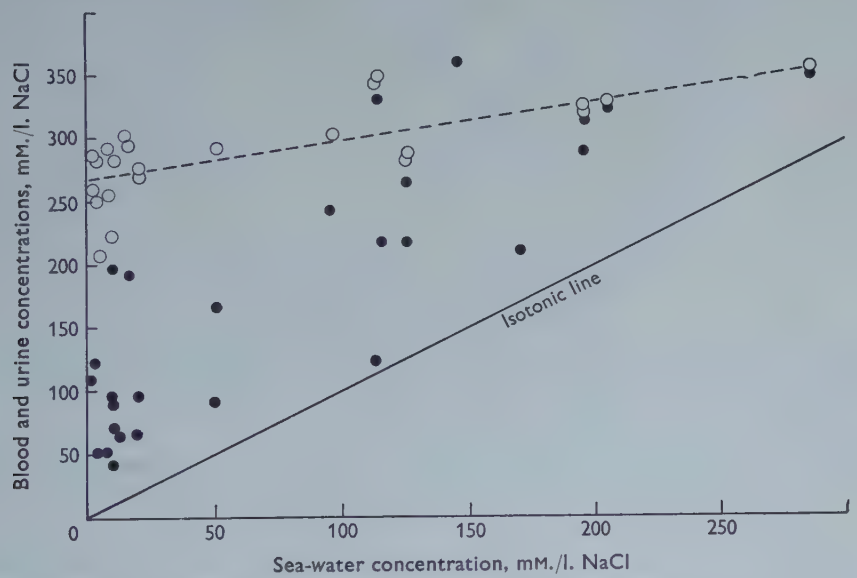

Fig. 1. The relation between the concentration of blood, urine and medium in *G. duebeni*. Specimens fully acclimatized to their medium. Circles = blood concentration, solid circles = urine concentration.

A plot of blood concentration against the lowest urine concentration observed for each fully acclimatized individual indicates that there is a general correlation between blood and urine concentrations (circles) Fig. 2. However, it should be noted that animals exposed to dilute media for only a short time before determination (less than 10 hr.), and therefore not yet fully acclimatized, tend to produce dilute urine even though the blood concentration is high (solid circles, Fig. 2).

G. duebeni adapted to fresh water produces urine whose concentration is only about one-third to one-fifth that of the blood. The absolute concentration of this urine is,

nevertheless, still markedly greater than that formed by the fresh-water species *G. pulex* (Table 1)

When *G. pulex* is acclimatized for 4–7 days to dilutions of sea water in the range of concentration 90–140 mM./l. the urine produced is little more concentrated than it is in fresh water. In consequence the concentration of the urine is markedly less than that of the medium (Fig. 3).

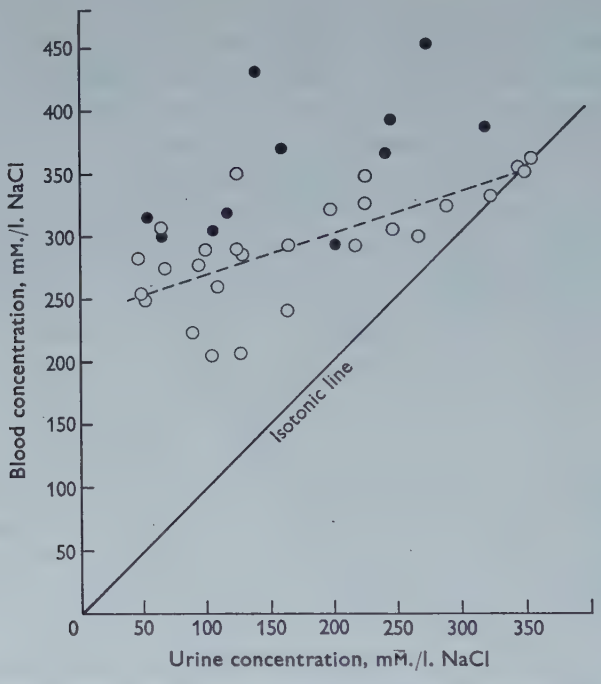

Fig. 2. The relation between blood and urine concentrations in *G. duebeni*. Circles = animals acclimatized to the medium for 3 days or longer. Solid circles = animals acclimatized to their medium for less than 10 hr. following previous adaptation to a high salinity.

Table 1. *Comparison of blood and urine concentrations of* Gammarus duebeni *and* G. pulex *acclimatized to media less concentrated than* 14 mM./l. NaCl

	G. pulex				*G. duebeni*			
	Mean	σ	Range	No.	Mean	σ	Range	No.
Blood concentration as mM./l. NaCl.	152	± 6	144–157	(6)	255	±37	205–305	(11)
Urine concentration as mM./l. NaCl.	27	±15	5–50	(13)	83	±28	48–142	(21)

No assessment of the part played by the excretory organ in the osmoregulation of an animal can be made without knowledge of the rate of urine flow. Direct determination of the urine flow by continuous collection of the urine produced was found to be technically rather difficult in the case of *G. duebeni*. An estimate of urine flow may be made, however, if the blood concentration and the water flux across the body surface are known, and if it is assumed that the net entry of water into the body

results solely from the differences between the diffusion of water into and out of the body.

The flux of water across the body wall will be proportional to the activity of the water on either side. A measure of the water activity is obtained by osmotic pressure determinations and activities can be expressed conveniently in terms of the concentration of water in equivalent NaCl solution (1 litre of pure water contains 55·5 mols H_2O and 1 litre of $\frac{N}{10}$ NaCl contains approximately 55·3 mols H_2O). The net rate of water movement across unit area of the membrane will be $K(C_0 - C_i)$, where K is

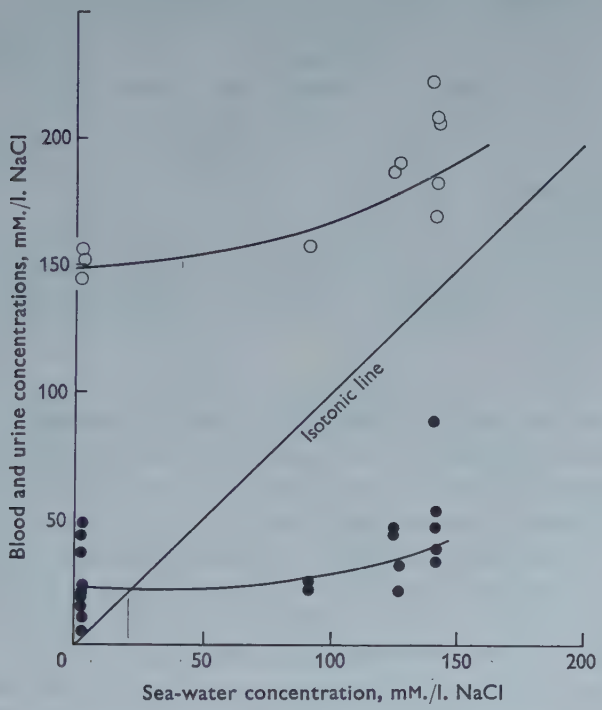

Fig. 3. The relation between blood, urine and medium concentrations in *G. pulex*. Circles = blood concentration, solid circles = urine concentration.

a constant and C_0 and C_i are the concentrations of water on the two sides. The proportion of net water movement to influx will therefore be $(C_0 - C_i)/C_0$. If $t_{\frac{1}{2}}$ is the half-time of exchange (in minutes) of body water the percentage of body water exchanged in 1 min. is given by the expression $(100 \ln 2)/t_{\frac{1}{2}}$. The daily net entry of water into the body will be:

$$100.M.\frac{C_0 - C_i}{C_0} \ln \frac{2}{t_{\frac{1}{2}}}, \qquad (1)$$

where M is the number of minutes in a day. If the net entry of water into the body involves a bulk-flow component the actual rate of urine flow will be greater than that calculated. No exact measure of urine flow may therefore be obtained, but the formula gives an estimate of the minimum urine flow in terms of percentage of body water per day.

The half-time for water exchange was determined as follows. Animals were acclimatized for 64 hr. in tritiated Cambridge tap water and were then placed in unlabelled tap water. Aliquots of this were taken at intervals and counted. The temperature was 20° C. The half-time for exchange of water was derived from semi-logarithmic plots of $T_e - T_t$ against time, where T_e is the count at equilibrium and T_t the count at time t. The results are given in Table 2 together with calculated values for urine flow per day as a percentage of body water.

Table 2. *Comparison of rates of exchange of body water and calculated urine volumes in* Gammarus pulex *and* G. duebeni

	G. pulex				*G. duebeni*			
	Mean	σ	Range	No.	Mean	σ	Range	No.
$T_{\frac{1}{2}}$ (min.) for T_{20} exchange with body water	11·6	3·0	7–15	(6)	13·9	1·2	12·25–14·75	(4)
Urine volume as % body water per day calculated from mean $T_{\frac{1}{2}}$ and blood concentrations	46				71			

Urine volumes calculated from $(C_0 - C_i)/C_0 \ln 2/T_{\frac{1}{2}} M$ 100 (see text), where C_0 is the concentration of water in the medium (55·5 M./l. and C_i is the concentration of water in the blood. C_i for *G. pulex* is 55·2 M./l. and for *G. duebeni* is 55·0 M./l.

Values for urine flow given in the literature are usually presented in terms of percentage of body weight per day. Values for the water content of *Gammarus* species qnoted by Vinogradov (1953) range from 74 to 83 % of the body weight. The calculated urine flow in percentage of body weight per day will therefore be of the order of 37 and 56 in *G. pulex* and *G. duebeni*, respectively.

These values are very high in comparison with the urine flow reported for animals such as: *Eriocheir sinensis* 4% body wt./day Scholles (1933), *Potamobius* 4% per day (Scholles 1933), *Potamon niloticus* 0·05–0·6% per day (Shaw, 1959*b*), *Astacus fluviatilis* 8·2% per day (Bryan, 1960*a*), but are not unexpected in view of the small size of the gammarids and consequent high surface/volume ratio. Since urine is formed from the blood it is clear that it is only by the formation of hypotonic urine that a very rapid rate of ion loss from the body can be avoided. Shaw & Sutcliffe (1961), using 40 mg. animals, found a rate of loss of sodium to distilled water of 0·76 μM./hr. in *G. duebeni* previously acclimatized to 2% sea water and 0·17 μM./hr. in animals acclimatized to 0·25 mM./l. NaCl. In the present experiments animals of this species acclimatized to Cambridge tap water and then placed in distilled water showed a loss of ^{22}Na equivalent to 6·1% (± 1·6%, $n = 5$) total body sodium/hr. over a 5 hr. period. The loss of sodium via the urine from animals acclimatized to Cambridge tap water, calculated on the basis of a 40 mg. animal, a urine flow of 71% of the body weight per day and a urine concentration of 83 mM./l., is 2·4 μM./day or 0·10 μM./hr. This calculated value for urinary sodium loss is equivalent to 13 and 60% respectively of the total losses recorded by Shaw & Sutcliffe for animals in 2% sea water and in 0·25 mM./l. NaCl. Now the urine produced by *Gammarus* is hypotonic to the blood at these concentrations, but it is clear that if it were isotonic the urinary salt loss would form a considerably larger proportion of the total loss than is observed for *Eriocheir* and *Potomon*.

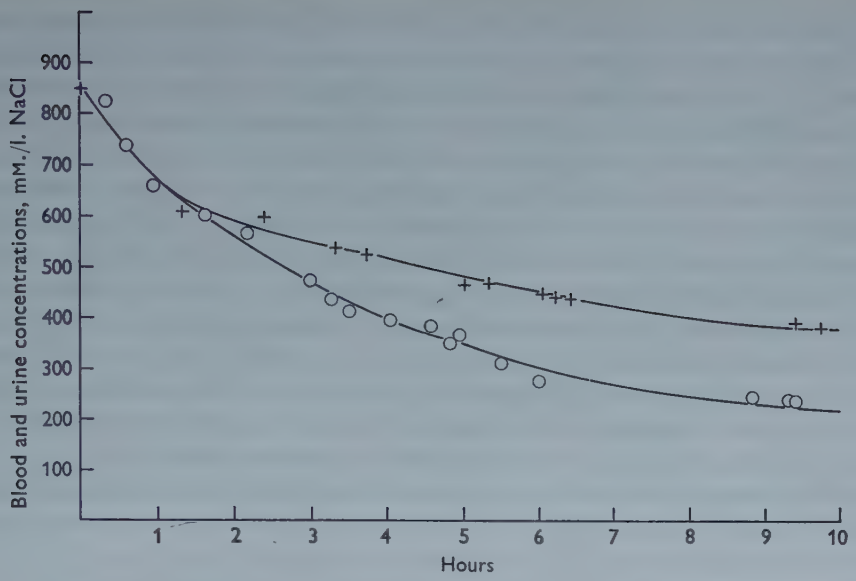

Fig. 4. Blood and urine concentrations of a specimen of *G. duebeni* transferred from 160% sea water to Cambridge tap water at zero time. ○ = urine, + = blood.

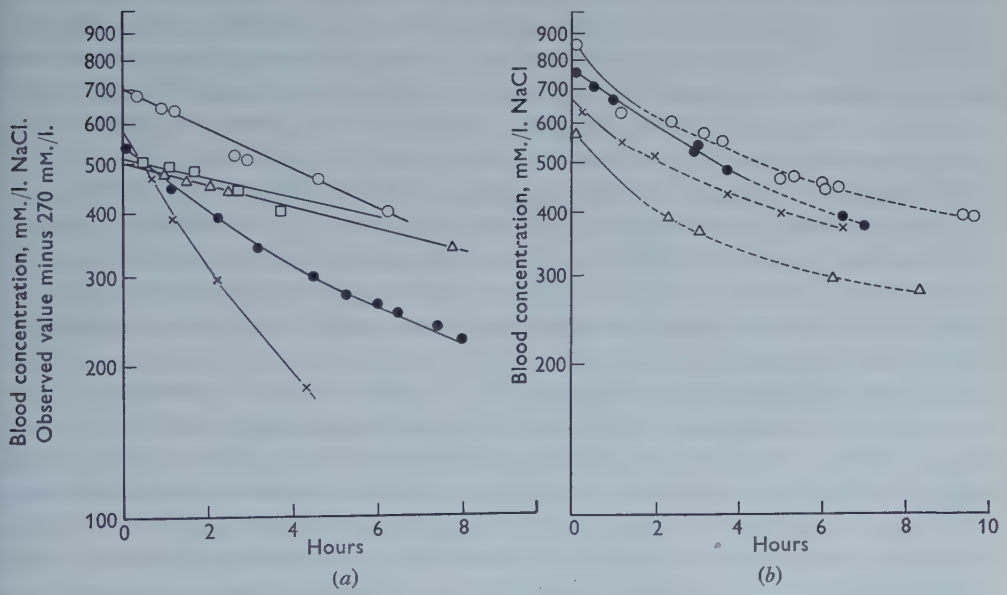

Fig. 5*a*. Semi-logarithmic plot of fall in blood concentration against time of *G. duebeni* transferred from 160 to 190% sea water to 50% sea water. 270 mM. (50% sea water) has been subtracted from the observed blood values prior to plotting in order that a direct comparison be made between this series of curves and those in Fig. 5*b*. † Animal died.

Fig. 5*b*. Semi-logarithmic plot of fall in blood concentration against time of *G. duebeni* transferred from 110 to 160% sea water to fresh water. Solid lines indicate blood conccentration at times when the urine is isotonic with the blood. Dotted lines indicate blood concentration when urine is hypotonic to the blood.

The rate of loss of sodium to distilled water is comparatively high and this suggests that rapid changes will occur in the blood concentration following a change in the concentration of the medium. *G. duebeni* may be readily acclimatized to 175% sea water and withstands direct transference from this medium to fresh water. Following transference from 110 to 160% sea water to fresh water there is, as expected, an initial rapid fall in the blood concentration. The urine is isotonic with the blood initially but usually after 1½–2 hr. the urine becomes hypotonic (Fig. 4). The urine becomes hypotonic despite the fact that at this time the blood may be up to twice as concentrated as the highest blood concentration at which animals fully adapted to their medium produce a hypotonic urine. Three further repetitions confirmed this effect (Fig. 5*b*). Five *G. duebeni* transferred from 160 to 190% sea water to 50% sea water, at both of which concentrations isotonic urine would be expected in fully acclimatized animals, all failed to form hypotonic urine within the next 4½–8 hr. (Fig. 5*a*). Two of these animals re-tested after 24 hr. were still producing isotonic urine.

The blood concentration falls in a logarithmic manner in animals which do not form hypotonic urine. In those that do form hypotonic urine the blood concentration falls somewhat less rapidly (Fig. 5*a*).

DISCUSSION

On the basis solely of studies on different species of crayfish it has long been accepted that an antennary gland with a long excretory tubule is potentially able to produce hypotonic urine. Additional support for this contention is now provided by the fact that hypotonic urine is formed by the two gammarids, *G. duebeni* and *G. pulex*, both of which have been shown by Hynes (1954) to have excretory tubules which are long in comparison with those of the more marine species *G. locusta*.

The fact that *G. duebeni* produces hypotonic urine when in brackish water also illustrates the fact that the capacity to form hypotonic urine is not the prerogative of fresh-water species. Furthermore, since an essentially brackish-water species forms hypotonic urine this ability cannot be regarded as being one of the later evolutionary stages in the physiological adaptation of fresh-water species to their environment as was suggested by Beadle (1943).

It has been postulated that the production of hypotonic urine by animals in fresh water results in some saving in the energy expended in osmoregulation (Potts, 1954) though the basis of this view has recently been criticized by Croghan (1961). Potts also produced evidence to show that the production of hypotonic urine in brackish water would result in little energy saving. It may be presumed therefore that hypotonic urine formation by *G. duebeni* has some other functional significance. It is suggested here that the production of hypotonic urine may subserve two important functions in *G. duebeni*.

(1) Ions already within the body are retained and hence less burden is placed on the mechanisms reponsible for active uptake at the body surface.

(2) The formation of hypotonic urine will tend to diminish the rate of fall in the blood concentration following any sudden dilution of the medium. Time is thereby gained for the osmotic adjustments which must be made by the general body cells when the blood concentration is changed.

These two functions may be expected to be of particular importance in small

species maintaining themselves markedly hypertonic to their medium, or rather in hypertonic species whose urine volume per day represents a large proportion of the body volume. (Small species will naturally have a greater urine volume/total volume ratio than larger forms of similar blood concentration and surface permeability.)

It is calculated from the half-time of exchange of tritiated water that the urine flow of *G. duebeni*, when in fresh water, is approximately equivalent to 70% of the total body water per day. Urine is formed from the blood and, in most Crustacea so far studied, the blood accounts for some 25–50% of the total water of the animal. Thus if an isotonic urine were to be formed by *G. duebeni* in fresh water, the daily salt loss by this route would be at least equivalent to the entire salt content of the blood and might be more than twice this amount. Correspondingly smaller amounts of salt would be lost in brackish water. Production of hypotonic urine will diminish this loss. Crabs such as *Eriocheir sinensis* and *Potomon niloticus* which produce isotonic urine when they are in fresh water have comparatively low rates of urine production and their rate of salt loss via this route is small relative to their total loss. Selection pressure to form a hypotonic urine is therefore presumably less intense in these forms. Beadle & Cragg (1940) found that *G. duebeni* maintained its blood increasingly more hypertonic to the medium in the range 2–50% sea water but was effectively isotonic at concentrations above 50–60% sea water. In the present study hypotonic urine formation has not been observed in animals fully adapted to media more concentrated than 50% sea water. Fig. 2 illustrates that the urine is progressively more dilute as the blood concentration is lowered.

It is patent that if the permeability of the body surface remains unchanged the urine flow will increase with the gradient of osmotic concentration maintained between blood and medium. Since the most dilute urine is formed when the urine flow is high, it is clear that the concentration of the urine is not directly related to the time taken for the urine to pass down the excretory tubule. Some form of active control must therefore regulate the urine concentration. It has repeatedly been shown that a lowering of the blood concentration brings about an activation of the processes responsible for the uptake of ions at the body surface of fresh-water and brackish-water animals (Shaw, 1958, 1959*a*, 1960, 1961; Shaw & Sutcliffe, 1961; Lockwood, 1960). The relation between urine and blood concentrations (Fig. 2) suggests that some similar mechanism may regulate the urine concentration. However, the urine concentration cannot be linked solely to the blood concentration. This follows from the fact that when specimens of *G. duebeni* are acclimatized to a high salinity and are then transferred to fresh water they start to produce hypotonic urine when the blood concentration has fallen only to a level equivalent to 100% sea water (Fig. 3), twice the concentration at which hypotonic urine formation would be expected in animals fully acclimatized to their medium. Fig. 2 also illustrates that this is a general feature in animals exposed for only short periods to dilute media, the urine usually being markedly more dilute in these animals than would be expected in fully acclimatized animals having the same blood concentration. Specimens of *G. duebeni* transferred from 160 to 190% sea water to 50% sea water did not produce hypotonic urine. It cannot therefore be postulated that the stimulus causing animals to produce hypotonic urine when transferred from 110 to 160% sea water to fresh water is merely a rapid fall in the blood concentration. The precise nature of the stimulus is far from clear and requires further investigation.

Comparison of the falling blood concentrations of animals transferred from 110 to 160% sea water to fresh water and from 160 to 190% sea water to 50% sea water illustrates that the production of hypotonic urine by the former decreases the rate of change of blood concentration (Fig. 5). The fall in blood concentration of the animals with isotonic urine approximates to a logarithmic curve, as would be expected if the permeability remained constant.

Shaw & Sutcliffe (1961) have investigated the rate of loss of sodium in *G. duebeni* acclimatized to different media at the lower end of their salinity tolerance range. They find that animals fully acclimatized to 2% sea water have a higher rate of sodium loss from the body than have animals acclimatized to 0·25 mM./l. NaCl. They calculate that the difference in the rates of loss could be accounted for if the animals in 2% sea water produced isotonic urine and if those in 0·25 mM./l. NaCl produced very dilute urine, provided that the urine flow was equivalent to approximately 7·4% of the body weight per hour. The present results indicate that the urine is already hypotonic in 2% sea water and that the urine flow is about 3% body water/hr. in fresh water. Some indirect support seems therefore to be provided for an alternative suggestion of Shaw & Sutcliffe that the permeability of the body surface may be varied in animals acclimatized to very dilute media. A critical study of the water fluxes of animals adapted to dilute media should indicate whether or not the regulation of water entry plays any part in this process.

The fresh-water species *G. pulex* is not normally exposed to saline media and it lacks the capacity to vary the urine concentration when that of the medium is raised. In consequence, in media more saline than about 20–30 mM./l. the urine is hypotonic not only to the blood but also to the medium; it is possible that as in the case of the crayfish (Bryan 1960*b*) the urine concentration may rise at very high concentrations of the medium. Urine formation can therefore play little part in the restriction of changes in the blood concentration of *G. pulex* in saline media. Though the production of urine hypotonic to the medium will not in itself result in a rise in the concentration of the blood as long as the animal is hypertonic to its medium, the maintenance of the blood concentration will depend to a large extent on the degree to which the active uptake of ions at the body surface can be suppressed.

The ability of *G. duebeni* to vary the concentration of the urine gives this animal a twofold means of regulating its blood concentration, which is presumably of some importance in the fluctuating salinities inhabited by this species.

If the excretory organ is to play any effective part in regulating the blood concentration following a change in the medium, the urinary salt loss must constitute an appreciable proportion of the total loss and it must be possible for the animal to effect rapid changes in the urine concentration. It is not surprising therefore, that changes in the gradient of concentration between urine and blood in *G. duebeni* can be varied markedly within 2 hr. of a change in the concentration of the medium.

It is hoped to extend the present study to cover urine production in *G. lacustris*, *G. zaddachi*, *G. salinus* and *G. locusta*. Preliminary studies indicate that the first three of these species produce hypotonic urine when they are in dilute media.

SUMMARY

1. A study has been made of the relation between blood, urine and medium concentrations in the two amphipod Crustacea *G. duebeni* and *G. pulex*.

2. *G. duebeni* produces urine hypotonic to the blood but hypertonic to the medium when it is in media more dilute than 50% sea water.

3. *G. pulex* forms urine which is hypotonic both to blood and medium when in 2–20% sea water.

4. *G. duebeni* begins to form hypotonic urine within 2 hr. of transference from 110 to 160% sea water to fresh water. Hypotonic urine formation begins in these circumstances when the blood concentration is up to twice that at which hypotonic urine is formed by animals fully adapted to their medium.

5. It is concluded (*a*) that the concentration of urine produced by *G. duebeni* is not dictated solely by the absolute level of the blood concentration; (*b*) that the formation of urine hypotonic to the blood in a brackish-water animal functions primarily as a means of conserving ions in the body; (*c*) that the ability to regulate the concentration of the urine with rapidity will be important in an animal living in environments of fluctuating salinities.

I am most grateful to Mr T. Warwick who collected many of the animals for me and who loaned me some specimens of *Gammarus* species varified by Mr D. M. Reid.

I am also indebted to Drs Shaw and Sutcliffe for permission to examine the proofs of their paper on *G. duebeni* and *G. pulex*.

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OSMOTIC REGULATION IN THE CRAB-EATING FROG (*RANA CANCRIVORA*)

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INTRODUCTION

Among the lower vertebrates the amphibians are probably the group in which mechanisms of osmotic and ionic regulation have been most carefully studied and hence are best understood. As is often the case, however, attention has been paid to only a few of the many species of the group. One result of the relatively narrow range of amphibians investigated has been the development of a firm belief that amphibians in general cannot survive for more than a few hours in external media more concentrated than about 300–350 milliosmolar (equivalent to a salinity of 9–11‰) (Adolph, 1933; Bertin, 1920; Brunacci, 1914; Durig, 1901; Duval, 1928; Overton, 1904; Przylecki, 1922; Rey, 1938). This belief ignores repeated observations in many parts of the world of the occurrences of a variety of amphibia, virtually all anurans, in brackish and even marine environments (Neill, 1958; Ruibal, 1959; Schmidt, 1957).

None of these more or less euryhaline amphibia has been investigated with respect to salinity tolerance, osmoregulatory mechanisms, etc., with the sole exception of the variegated or green toad (*Bufo viridis*) of Europe and the Middle East. Adult *B. viridis* have been shown to tolerate, for periods up to at least a month, external salinities as high as 29‰ (coastal sea water usually has a salinity of about 31‰). This amazing resistance to hypertonic media apparently is based neither upon skin impermeability nor upon drinking of external environment (Stoicovici & Pora, 1951). Excepting a study of the role of the nervous system in salinity adaptation in this form (Pora & Stoicovici, 1955), nothing conclusive is known about the physiological mechanisms used by *B. viridis* in accomplishing this feat.

One of the most consistent sources of reports of anurans in saline environments has been the tropics of south-east Asia. The most frequent and spectacular reports concern the crab-eating frog (*Rana cancrivora*) and several of its close congeners, including the form which systematists consider *R. cancrivora's* closest relative, the tiger frog (*Rana tigerina*) (Neill, 1958).

During the summer of 1960, we had an opportunity to visit South Viet Nam and Thailand to investigate in detail the validity of the recurring reports of unusual salt tolerance in these two species. We confirmed the reports for *R. cancrivora*, but not for *R. tigerina*. The present paper describes the unexpected physiological mechanisms by means of which *R. cancrivora* osmoregulates. Data showing that *R. tigerina* behaves ike an ordinary frog are also included.

The major results of this work have been briefly summarized by Gordon, Schmidt-Nielsen & Kelly (1961).

MATERIALS AND METHODS

R. cancrivora Gravenhorst is a small- to medium-sized frog, most adults ranging in weight from about 30 to 50 g. It is difficult to separate morphologically from several closely related forms. True *R. cancrivora* appears to be restricted to coastal lowland areas between southern South Viet Nam and southern Thailand. Several forms from adjacent areas such as the Philippines, Malaya and Indonesia have been considered to be *R. cancrivora* at one time or another, but, excepting possibly the Philippine frogs, these other forms are probably distinct species (Bourret, 1942; Taylor, personal communication).

Both adults and partly grown tadpoles (only a few at limb bud stage) of *R. cancrivora* were collected in the vicinity of the village of An Hin, in the mangrove belt along the north shore of the Gulf of Thailand, about 60 miles south-east of Bangkok, Thailand. Adults are nocturnal and were common after dark under and among the houses of the village, and also among the surrounding mangroves. They were easily captured by hand, in the light from a head lamp. Tadpoles were abundant in water-filled ditches. dug by the villagers a short distance above high-tide marks. The salinity of the water in these ditches varied with the time since the last rain. We were unable to obtain ripe eggs and sperm of *R. cancrivora* as the breeding season for this frog had apparently passed by the time we arrived (*R. cancrivora* breeds in June in the Philippines (Alcala, 1955)).

R. tigerina Daudin closely resembles *R. cancrivora*, but is a medium to large frog, adults often weighing more than 150 g.

We follow Taylor & Elbel (1958) in using the spelling *tigerina* for the specific name of this form. *Tigerina* occurs in lowland areas throughout continental south-east Asia from at least southern China to Malaya and west to Burma (Bourret, 1942) and is one of the commonest frogs in flooded rice fields and roadside ditches.

Adult *R. tigerina* were purchased from local farmers and fishermen near Nhatrang, about 250 miles north-east of Saigon, South Viet Nam. They are a common food item in this area, hence were readily available—though most abundantly after rains. Our visit to Viet Nam took place in June and early July and most adults purchased contained ripe eggs and sperm.

Solutions used as external media for the frogs were, in Viet Nam, dilutions of coastal sea water ('100% sea water', $\Delta = 2{\cdot}0^{\circ}$ C., salinity = 36‰). In Thailand, the salinity of the near-shore sea water was somewhat variable and adequate quantities of sea water were not easily obtainable for use in Bangkok. We therefore mostly used solutions of sun-dried sea salt. These solutions were standardized so that '100% laboratory salt water' (l.s.w.) had $\Delta = 1{\cdot}9^{\circ}$ C., salinity = 35‰. Several parallel experiments were carried out with groups of adult *R. cancrivora* using equal osmotic concentrations of laboratory salt water and diluted Gulf of Thailand sea water (natural sea water, n.s.w.).

Unless noted otherwise, the following series of observations were made on groups of five or more frogs of each species, variously acclimatized to different salinities.

All experiments were carried out at room temperature, which was 27–30° C., both in Thailand and Viet Nam. Frogs were not fed. No indications of physiological differences between frogs of the two sexes were noted.

(1) Survival and change in body weight following transfers from fresh water to various dilutions of sea water, or from one dilution of sea water to another. Frogs were kept, for the duration of such experiments, in small, tared, lightweight covered plastic cups so that they could be weighed without being directly handled. These cups were perforated to allow circulation of water and air. Weighings were done on a 500 g. torsion balance with a sensitivity of 0·05 g. Repeated weighings of single frogs, following immersion in water, draining and removal of excess water by wiping drops from the outside of the box, usually agreed within ±0·1 g.

(2) Plasma samples were obtained by centrifugation of blood collected with heparinized glass capillary tubes (*R. cancrivora*) or heparinized syringes (*R. tigerina*) directly from the hearts of frogs. Determinations were:

(*a*) Freezing-point depression (Δ). For *R. cancrivora* Δ was determined to a precision of ±0·01° C. on samples of $\sim 10^{-4}$ mm.^{3} using the apparatus of Ramsay & Brown (1955). In Nhatrang dry ice was unavailable, therefore for *R. tigerina* Δ was measured on several ml. of pooled blood. The samples were placed in an ice-salt mixture at about −6° C. and their supercooling curves were followed by means of a precision thermometer calibrated to ±0·005° C. which was also used to stir the samples. This crude osmometer, calibrated against known standards, gave results accurate to ±0·02° C.

(*b*) Chloride was determined by a Volhard $AgNO_3$-SCN titration on duplicate samples of 0·050–0·100 ml. Average precision ±2 m-equiv./l.

(*c*) Sodium was determined by flame photometry on duplicate samples of 0·025–0·100 ml. diluted 100× with glass distilled water. Average precision ±5 m-equiv./l. These analyses were made only on plasma of *R. cancrivora* using samples which had been sealed into glass capillaries, then immediately frozen and shipped by air, frozen on dry ice, to California.

(*d*) Potassium. By flame photometry on same samples as Na. Average precision ±0·5 m-equiv./l.

(*e*) Urea. By the micro-diffusion technique of Conway. On duplicate 0·100 ml. aliquots of the same 100× diluted samples used for Na analyses. Average precision ±0·02 moles/l. Only traces of NH_3 in samples.

(3) On urine samples obtained, usually just before blood sampling, via polyethylene catheters inserted into the cloaca: Δ and Cl (both species); Na, K, urea (on *R. cancrivora* samples only). Techniques and precision as for plasma analyses.

(4) Electrical potentials and short-circuit currents across isolated pieces of belly skin using the apparatus of Ussing (1954). Ringer solutions made according to the formula given by Adrian (1956) were used on both sides of the isolated skins. The Cl concentration was adjusted to approximate measured plasma levels in our experimental frogs by varying NaCl concentration in the solutions. No Ringer solutions containing urea were used. A Triplett Model 631 portable VTVM was used for potential measurements, with two Beckman 39270 calomel electrodes, previously calibrated against one another. A Simpson Model 260 portable VOM was used with a 1·5 V. flashlight battery, a 72,000 Ω variable resistor and two Ag-AgCl electrodes

for short-circuiting the skins and for current measurements. Precision of potential measurements ±5 mV. Precision of current measurements ±0·01 mA. (490 mm.^{2} skin area across opening of cell). The isolated skins usually would survive for one to several hours, as shown by their electrical activity. However, only the first measurements made within 3–5 min. of the removal of the skin are reported because considerable changes have been shown to occur at the level of intracellular fine structure in surviving pieces of animal tissue isolated from living animals for periods even as short as 5 min. (Sjöstrand & Baker, 1958; Hanzon, Hermodsson & Toschi, 1959). In addition, important changes in electrical properties were noted in some of the skins we studied (cf. section of skin potentials).

(5) Evaporative water loss as measured by weight loss from frogs placed in a stream of air moving at known, constant velocity and with nearly constant relative humidity. Air stream velocities were measured with a Bacharach Model MRF 'Florite' air-velocity meter and relative humidities with a Taylor sling psychrometer. Frog body temperatures during the experiments were measured with a Yellow Springs Instrument Co. 'Telethermometer', Model 43 TD, using a small animal thermistor probe inserted several cm. into the gut via the cloaca.

(6) Preference of the animals themselves for concentration of their external medium. With *R. cancrivora*, groups of small frogs, with *R. tigerina*, individual large frogs, were placed in sand-floored boxes where shallow dishes filled with different sea-water concentrations were set with their edges flush with the sand surface. The positions of the frogs were noted at intervals up to about 24 hr. after the experiment began.

(7) Degree of development of artificially fertilized eggs placed in different concentrations of sea water. Groups of about twenty fertilized eggs were placed in each concentration and observed for about 48 hr.

RESULTS

Rana cancrivora

Survival and weight changes

Data on survival and percentage changes in body weights of adult frogs transferred to various concentrations of external medium from their natural habitat and following various acclimatizations are presented in Figs. 1–4.

Independent of initial state of acclimatization, frogs transferred to concentrations of either natural sea water (n.s.w.) or laboratory salt water (l.s.w.) of up to 50% (18‰ salinity), survived for 7–12 days with almost no mortality.

Acclimatization to elevated external concentrations improved survival at still higher concentrations. Frogs transferred from fresh water to 70% l.s.w. died within 12 hr. Acclimatization to 50% l.s.w. for 2–5 days enabled one frog out of five transferred to 60% l.s.w. to survive until observations ceased after 8½ days, while the four other frogs in this group survived for 2–4 days. Direct transfers from 50 to 80 or 100% n.s.w. or l.s.w. were uniformly fatal, but, at least for the 80% transfers, only after longer periods of time than for the fresh water to 70% transfers.

An estimate of the maximal sea-water concentration which *R. cancrivora* can tolerate indefinitely is obtainable from the frogs used for the experiment described in Fig. 4. Eight frogs were kept in 40% l.s.w. for three days, in 60% for another 3 days, then

finally transferred to 80% l.s.w. (28‰ salinity). Six of the eight frogs survived a sequence of 80% for 3 days, 64% for 1 day, then 80% for another 4 days. It is possible that such slowly acclimatized adult *R. cancrivora* or animals in their natural surroundings can indefinitely tolerate still higher salinities as there was a slight trend toward recovery of original body weight in the six surviving frogs toward the end of the experimental period.

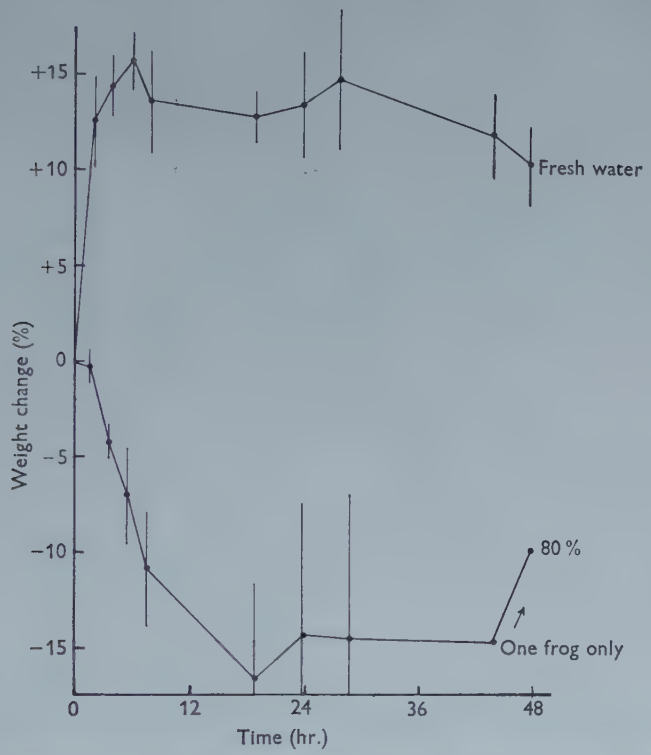

Fig. 1. Mean percentage weight changes in groups of *R. cancrivora* taken directly from their natural habitat and transferred either to fresh water or 80% l.s.w. (salinity 28‰). Vertical lines indicate ± 2 S.E. of the mean in this and all other figures.

Major changes in body weight which occurred after transfers were completed within about 24 hr. Changes in weight of up to 20% were tolerated and there was usually little sign of return to original weights (allowing for starvation effects in the interim) over periods of about a week. The direction taken by changes in body weight following transfers to media significantly different from initial body-fluid concentrations (see below) was apparently determined by the osmotic gradients established. This is most clearly shown in Figs. 1 and 4. The skin of *R. cancrivora* therefore appears to be readily permeable to water. The persistence of weight changes over long periods indicates the possibility that this frog does not drink the external medium even after severe dehydration.

The fairly synchronous cyclic (approximately 24 hr. duration) fluctuations in body weights shown in several graphs, especially Fig. 2, is possibly due to similarly cyclic accumulation of urine and emptying of the bladder. Another possibility is periodic

drinking of external medium followed by periods of slower water loss. The peaks of these fluctuations occurred about mid-day, the low points during the night. All series of weight measurements shown in Fig. 2 were carried out concurrently.

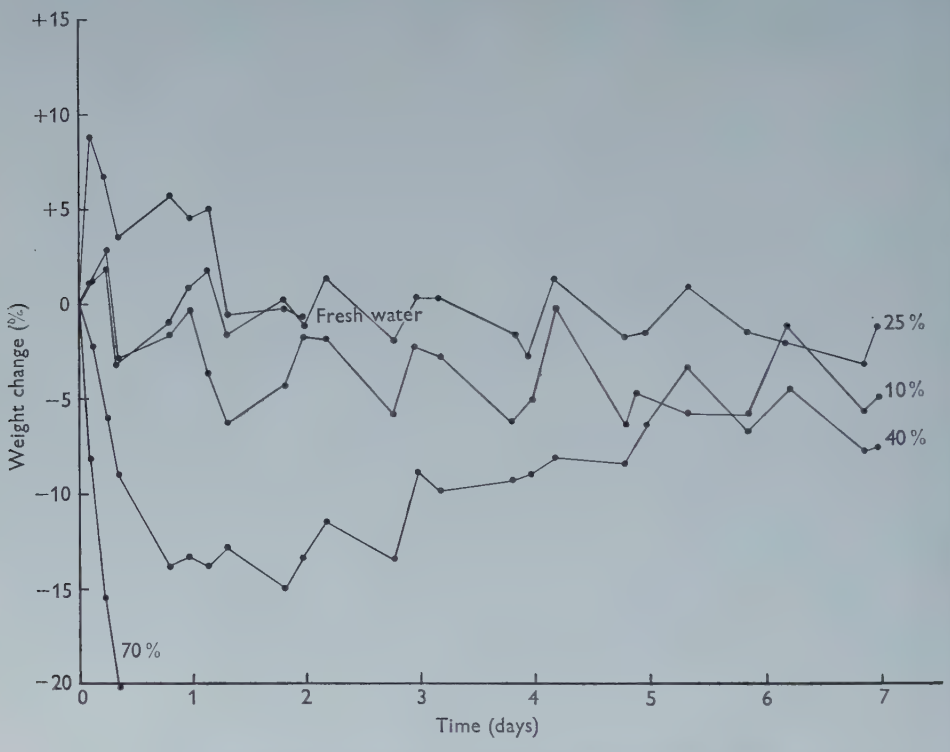

Fig. 2. Mean percentage weight changes in groups of *R. cancrivora* acclimatized to fresh water for at least 2–3 days, then transferred to fresh water (controls), 10, 25, 40 and 70 % l.s.w. S.E.'s of means similar to those shown in Fig. 1, but omitted from this figure for clarity.

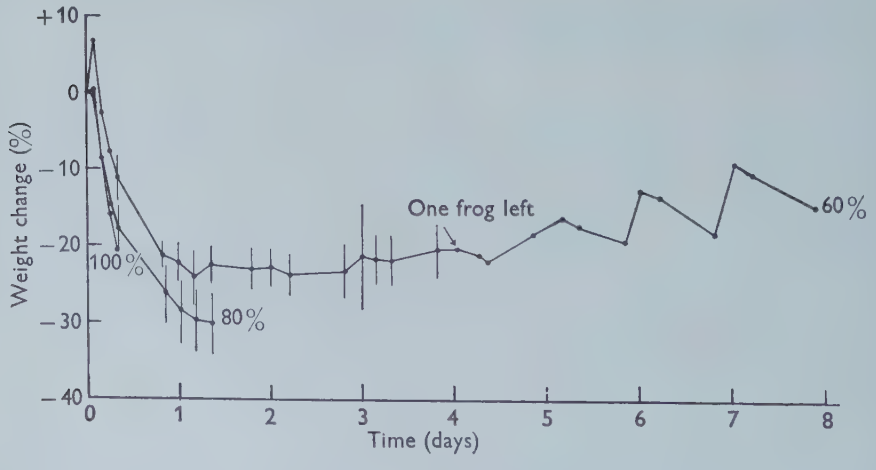

Fig. 3. Mean percentage weight changes in groups of *R. cancrivora* acclimatized to 50 % l.s.w. for 2–3 days, then transferred to 60, 80 and 100 % l.s.w.

The tadpoles of *R. cancrivora* were studied with respect to survival only. They seem, however, to be even more unusual than their parents.

The salinity of the water in the ditches in which the tadpoles live apparently fluctuates widely. Values as high as 35‰ and as low as 23‰ were measured during our brief observation period. Literally thousands of tadpoles were present in these ditches.

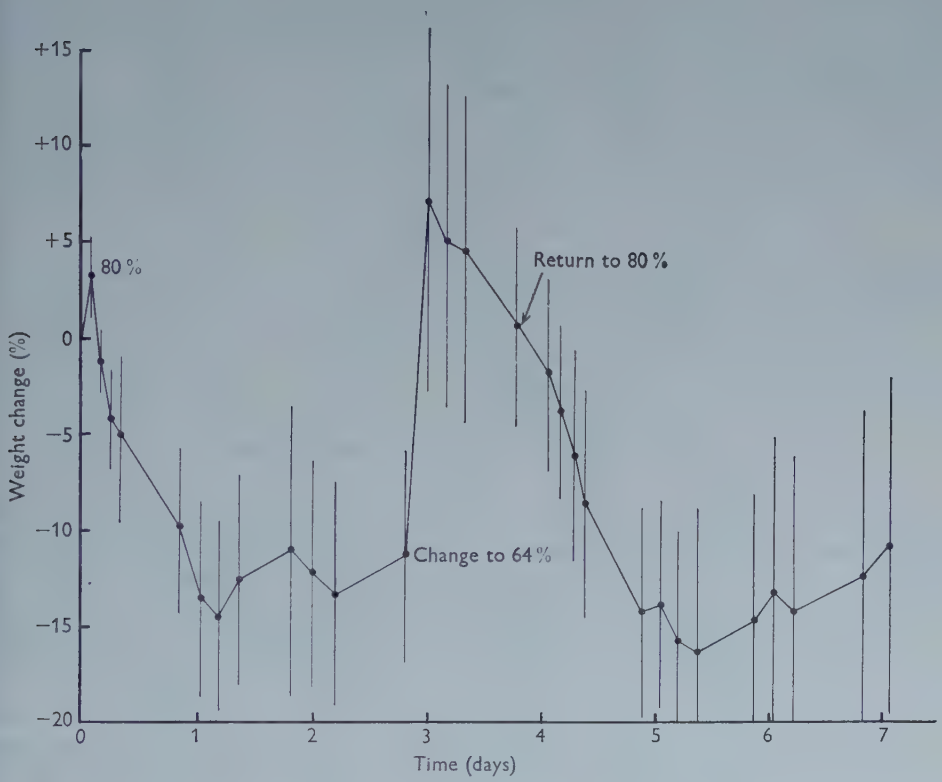

Fig. 4. Mean percentage weight changes in a group of *R. cancrivora* acclimatized for 3 days to 40 % l.s.w. and for another 3 days to 60 % l.s.w. They were moved to 80 % l.s.w., and after 3 more days in 80 %, changed to 64 % for 1 day, then returned to 80 % for remainder of experiment.

In transfer experiments a clear difference in responses to l.s.w. and n.s.w. was found. Tadpoles transferred from 23‰ salinity ditch water to 33‰ n.s.w. all survived for at least 5 days. This survival occurred despite the fact that evaporation increased the concentration in the tadpole's container to 39‰ (~120% n.s.w.) after only 3 days. However, tadpoles transferred to l.s.w. concentrations higher than 60%, whether from ditch water or tap water, all died.

Plasma and urine concentrations

Results of analyses for Δ, Cl, Na, K and urea of plasma and urine in variously acclimatized adult *R. cancrivora* are summarized in Table 1 and Figs. 5 and 6.

Probably the most striking facts emerging from these data are that *R. cancrivora* apparently is always somewhat hypertonic to its medium and that the greater part of

Table 1. *Plasma and urine concentrations in adult* Rana cancrivora

State of acclimatization	Concentrations [X ± S.E. (N)] Δ (m-osm./l.)	Cl (m-equiv./l.)	Na (m-equiv./l.)	K (m-equiv./l.)	Urea (mM./l.)
		Plasma			
FW, 2–7 days	290 ± 10 (6)	98 ± 10 (7)	125 ± 17 (5)	9 ± 1 (5)	40 ± 1 (5)
25 % l.s.w., 2 days	340 ± 15 (4)	122 ± 1 (4)	161 ± 13 (3)	8 ± 0·3 (3)	110 ± 1 (3)
50 % l.s.w., 2 days	—	179 ± 5 (5)	—	—	—
50 % l.s.w., 7 days	590 ± 10 (6)	155 ± 5 (6)	174 ± 5 (1)	6 ± 0·5 (1)	310 ± 20 (1)
50 % n.s.w., 2 days	560 ± 10 (1)	146 ± 2 (5)	191 ± 5 (4)	8 ± 0·8 (4)	280 ± 1 (4)
75 % l.s.w., 1 day	—	286 ± 8 (2)	—	—	—
80 % l.s.w., 7 days	830 ± 50 (5)	227 ± 9 (5)	252 ± 12 (4)	14 ± 0·5 (2)	350 ± 1 (4)
		Urine			
FW, 2–7 days	80 ± 5 (5)	5 ± 1 (5)	—	—	—
25 % l.s.w., 2 days	185 ± 30 (3)	9 ± 4 (4)	10 (3)*	2 (3)*	70 (3)*
50 % l.s.w., 2 days	—	33 ± 14 (4)	—	—	—
50 % l.s.w., 7 days	455 ± 20 (5)	30 ± 14 (6)	10 (5)*	9 (5)*	190 (5)*
50 % n.s.w., 2 days	—	10 ± 1 (5)	5 (4)*	42 (4)*	260 (4)*
75 % l.s.w., 1 day	—	273 ± 5 (1)	—	—	—
80 % l.s.w., 7 days	600 ± 70 (4)	12 ± 6 (4)	20 ± 5 (1)	21 ± 1 (1)	230 ± 20 (1)

* Pooled sample from indicated number of frogs.

the increase in body-fluid concentrations over fresh-water levels is due to urea (approximately 60% of the increased Δ in frogs in the highest salinities). Urea concentrations as high as 0·48 M (2·9%) have been measured in individual frogs. Frogs in fresh water for 3–5 days retain about 0·04 M. urea in their blood, a concentration about ten times higher than normal blood urea levels in fresh-water frogs (Forster, 1954).

Plasma Na and Cl also increase as the concentration of the external medium rises. Salt concentrations in the plasma of frogs in 80% l.s.w. are approximately twice those in the plasma of frogs in fresh water.

The urine of *R. cancrivora* was hypotonic to the blood independent of state of acclimatization. It was also uniformly low in monovalent inorganic ions. Considerable quantities of metabolites other than urea must also have been present in the urine as the total contribution of Cl, Na, K and urea to urinary Δ was only about 50% (Fig. 5). Urinary urea concentrations were high (0·23 M in one frog in 80% l.s.w.) and, while never more than about 60–70% of plasma levels, increased more or less proportionately to plasma levels (Fig. 6).

No measurements were made of rates of urine production, but the relative ease of obtaining urine samples from frogs even in 80% l.s.w., plus the magnitude of the daily cyclic fluctuations in weight of the frogs in most environments, make it seem probable that significant rates of production occur in frogs in all media.

Electrical potentials and short-circuit currents across the skin

Electrical potentials measured across isolated pieces of skin within 5 min. of removal from the abdomen of variously acclimatized *R. cancrivora*, are listed in Table 2. Ringer solutions of 75, 100 and 135% of the concentration used by Adrian (1956)

were used, respectively, on both sides of the skins of frogs from fresh water, 25% l.s.w. and 50% l.s.w.

The variability of the skin potential measurements is such that no particular relationship to acclimatization concentration is discernible. Excepting two frogs in 25% l.s.w. and one frog in 50% l.s.w. all skins generated lasting potentials such that their inner surfaces were positive. This would be consistent with a sodium pump operating in the usual direction for fresh-water frogs, that is, inwards. The three exceptional skin samples mentioned showed reversals in potential. The reasons for this remain obscure.

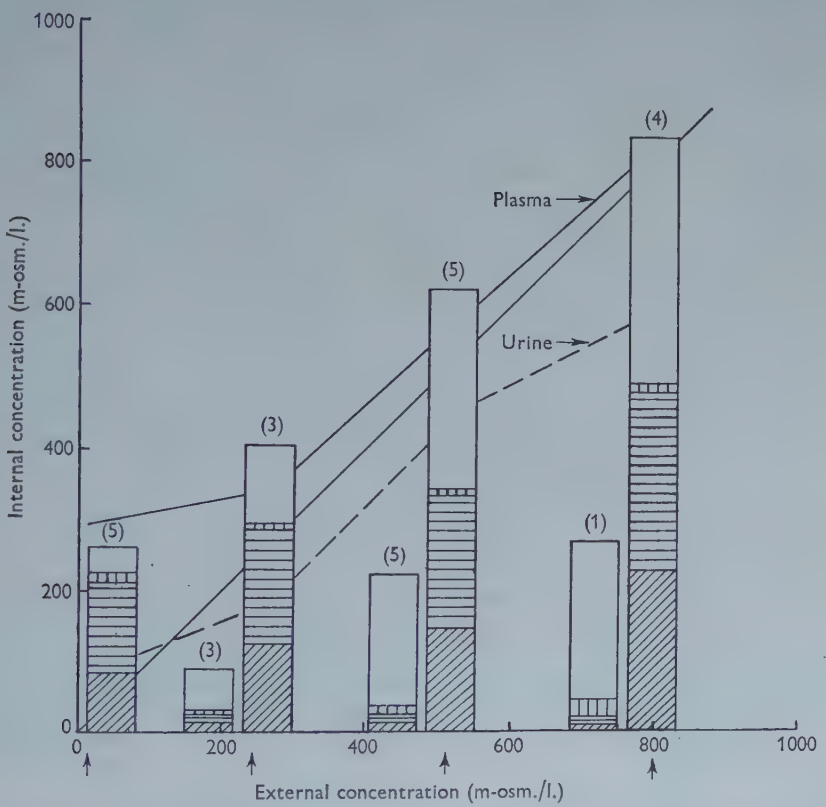

Fig. 5. Plasma and urine osmotic concentration, Cl, Na, K and urea in variously acclimatized *R. cancrivora*. All samples taken after at least 48 hr. acclimatization to each environment. Upper solid line (——) plasma osmotic concentration, middle line, line of equality between internal and external concentrations, lower dashed line (- - -) urine osmotic concentration. Right-hand bar in each pair plasma concentrations, left-hand bar urine concentrations. Four groups are frogs in fresh water and 25, 50 and 80% l.s.w. Arrows along abscissa mark actual acclimatization concentrations. ▨ = Cl^-, ▤ = Na^+, ▥ = K^+, □ = urea.

Short-circuit currents, expressed as current densities, measured across the same pieces of skin a few minutes after the potential measurements, are also summarized in Table 2. The relationship between skin short-circuit current and external concentration is not clear, though there are indications of a decrease in current in more concentrated media. Again, the measurements of persisting inward currents support the idea that the ion transport mechanism in the skin operates in the same direction

in frogs acclimatized to 50% l.s.w. as it does in frogs in fresh water. Since *R. cancrivora* in concentrated media appear to be in the same situation as other amphibia in fresh water, i.e. hypertonic to the medium, these results are not surprising. It would be very interesting to see if the addition of urea to Ringer solutions used for future studies of ion transport across isolated skins of *R. cancrivora* and other anurans would have any effects.

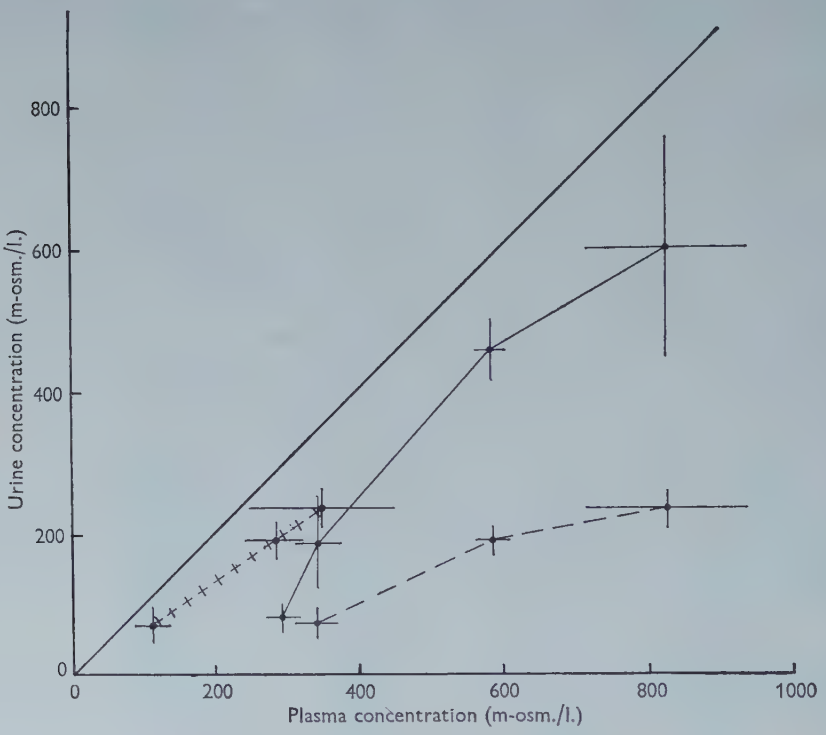

Fig. 6. Relationships between urine osmotic concentration and plasma osmotic concentration (——), urinary urea and plasma osmotic concentration (- - -), and urinary ureaand plasma urea (++) in variously acclimatized *Rana cancrivora*.

Table 2. *Potential and short-circuit current measurements in isolated skins of* Rana cancrivora

State of acclimatization	Skin potentials (mV) (inside of skin always positive)	Short-circuit currents (μA./mm.^{2})
FW	15–20, 20–25, 40–45, 70–75	0·53, 0·57, 1·39, 3·06
25 % l.s.w.	0, 15–20, 25–30, 60–65.	0·20, 0·41
50 % l.s.w.	15–20, 20–25, 25–30, 30–35, 35–40	0·12, 0·12, 0·16, 0·16, 0·45

A seemingly simple possible mechanism which would allow a frog to maintain a blood salt concentration lower than that of the external medium would be a reversal of the direction of ion transport by the skin. Our results indicate that *R. cancrivora* does not use such a mechanism in its physiological adjustment to saline environments. It should be noted, however, that the observed skin potentials and short-circuit

currents could have been caused by an active outward transport of Cl, rather than the usual inward transport of Na. The resolution of this problem awaits measurement of the ion fluxes across the skin.

Evaporative water loss

Evaporative water losses in a stream of air at 31° C., relative humidity of 55%, at a velocity of 5–7 m.p.h. are summarized in Fig. 7 for two groups of *R. cancrivora* of four each, acclimatized respectively to 10 and 40% l.s.w. Acclimatization periods were 7 days. Frogs were run in pairs, one from each acclimatization group, the four pairs consecutively in a single afternoon. Initial weights of the frogs were 12–20 g.

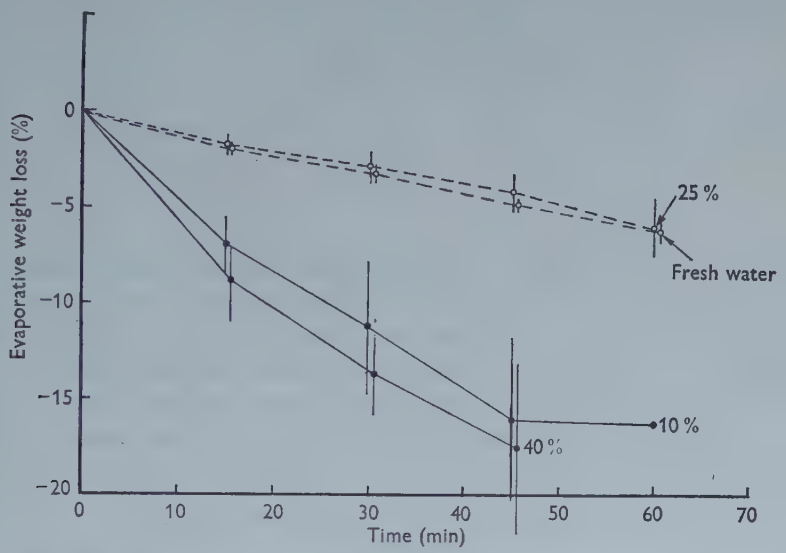

Fig. 7. Mean percentage weight losses due to water evaporation from groups of *R. cancrivora* (——) and *R. tigerina* (- - -) placed in moving streams of air at constant velocity, temperature and relative humidity. *Cancrivora* experiments done in Bangkok on groups acclimatized for several days to 10 and 40 % l.s.w. *Tigerina* experiments done in Nhatrang on groups similarly acclimatized to fresh water and 25 % n.s.w.

There are no statistically significant differences between the two groups. Apparently no important changes in permeability of the skin of *R. cancrivora* to water vapour occur in association with acclimatization to higher concentrations of external medium.

Body temperatures of these frogs were measured at intervals during these experiments. Within a few minutes after starting the fan which blew air over the frogs, their body temperatures dropped from only a degree or so below environmental temperature to the wet-bulb temperature. They remained at this level until the flow of air was stopped at the end of the experiment.

Environmental salinity preference

Two groups of five *R. cancrivora* each were acclimatized to fresh water and 50% n.s.w., respectively for, 3 days, then given their choice of 0, 25, 50, 75 and 100% n.s.w. The numbers of frogs present in each pan or on the sand floor outside the pans was noted

at intervals of 15–30 min. over the first 4 hr, then at intervals of about an hour (early morning hours excepted) until 24 hr. had passed.

There were eleven observation periods during the first 4 hr., thus fifty-five observations of the positions of individual frogs were made during this time for each acclimatization group. There was a total of twenty-two observation periods during the entire 24 hr., for a total of 110 position observations for individual frogs for each group. The percentages of these total numbers of possible occurrences accounted for by frogs in each of the possible environmental situations are summarized, for the two groups, in Table 3.

Table 3. *Environmental salinity preference by* Rana cancrivora

	Occurrence in each environment (% of total possible occurrences)											
	First 4 hr.						24 hr.					
Acclimatization group	0	25	50	75	100	Air	0	25	50	75	100	Air
FW	16	20	18	0	0	46	29	22	10	2	0	37
50% n.s.w.	20	38	13	0	2	27	33	39	7	0	2	19

The number of observations made is inadequate for a detailed analysis of environmental salinity preferences, but the data show that frogs in both acclimatization groups preferred external media no more concentrated than 50% n.s.w. The fresh-water frogs appeared to prefer sitting on the sand to everything else available, but otherwise showed no marked preference for a particular environmental concentration—as long as it was below 50%. The frogs acclimatized to 50% n.s.w. appeared to prefer sitting on the sand or in 25% n.s.w. initially, but shifted as time went on to fresh water and 25% n.s.w.

This species when pushed can, as an adult, tolerate at least up to 80% sea water. It apparently prefers a concentration of no more than about half that.

Rana tigerina

The results of our study of water balance and osmoregulatory mechanisms in *R. tigerina* are summarized in Figs. 7–9. The principal differences between our studies of *R. cancrivora* and *R. tigerina* are that, in the work on *R. tigerina*, we made no analyses for Na, K and urea, and that we were able to study the effect of environmental salinity on development of fertilized eggs.

Adult *R. tigerina* tolerate direct transfer and indefinite exposure (i.e. for at least 8 days) to all concentrations of n.s.w. up to 25% (salinity 9‰). 30% n.s.w. (salinity 11‰) is uniformly fatal within 24–48 hr., even after several days acclimatization to 25%.

Transfer of this species to any medium other than fresh water results in marked and rapid gains in weight. Return to about original weight takes place after 5 days (Fig. 8).

This transitory weight gain is probably due to swallowing of external environment. Phenol red was placed in the external medium in several experimental series. Quantities of the dye were present in the gut contents at the end of the experiments. The

intake of external medium will occur even in frogs with their mouths sewn shut. Perhaps the nostrils are used in this situation.

The contrast between the behaviour of *R. tigerina* and *R. cancrivora*, transferred to 25% n.s.w. and l.s.w., respectively, following several days in fresh water, is illustrated in Fig. 8.

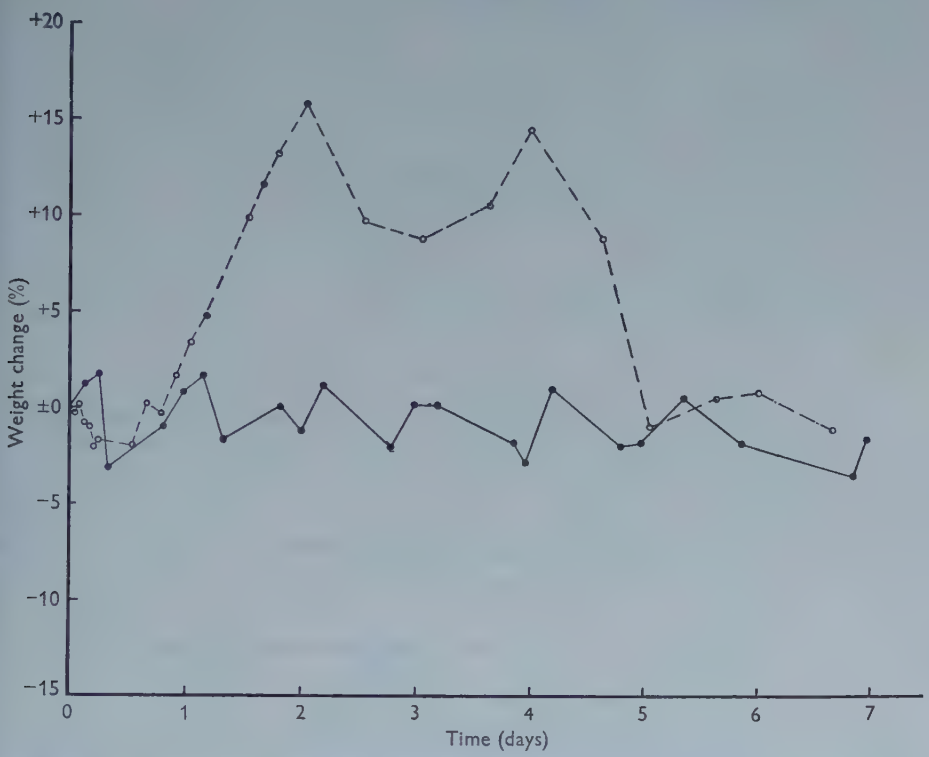

Fig. 8. Comparison between mean percentage weight changes in groups of *R. cancrivora* (——) and *R. tigerina* (- - -) each acclimatized to fresh water, then transferred to 25% sea water. S.E.'s of means omitted for clarity.

Data on Δ and Cl of plasma and urine in variously acclimatized *R. tigerina* are presented in Fig. 9. *R. tigerina* regulates its plasma concentrations fairly well up to 25% n.s.w., the kidney apparently being a very important organ in this effort. Urinary Δ and Cl both rise rapidly from levels far below those in the blood, in frogs in fresh water, to near equality with blood levels in frogs in 25% n.s.w. No measurements of rates of urine production were made, but undisturbed frogs, even in 25% n.s.w., usually accumulated considerable volumes of urine in their bladders.

As in *R. cancrivora*, no specific relationship was discernible between the magnitudes of the electrical potentials developed by isolated pieces of skin from variously acclimatized frogs and the concentration of the external medium. All such potentials were positive inward, ranging from 10 to 100 mV. in frogs in fresh water, 60 to 110 mV. in frogs in 10% n.s.w. and 25 to 40 mV. in frogs in 25% n.s.w. Isolated skins of *R. tigerina* usually survived for several hours. No reversals of potential occurred such a were noted above for a few *R. cancrivora* skins.

Measurements of short-circuit currents across isolated skins indicate that the skin also plays a part in the effort this frog makes in adjusting to saline media. Short-circuit current across the skin was only about 10% of fresh water values in frogs in 25% n.s.w. Mean values and ranges were 0·72 (0·45–0·94) μA./mm.^{2} for four frogs in fresh water, 0·46 (0·29–0·73) μA./mm.^{2} for four frogs in 10% n.s.w., and 0·08 (0·08–0·08) μA./mm.^{2} for two frogs in 25% n.s.w.

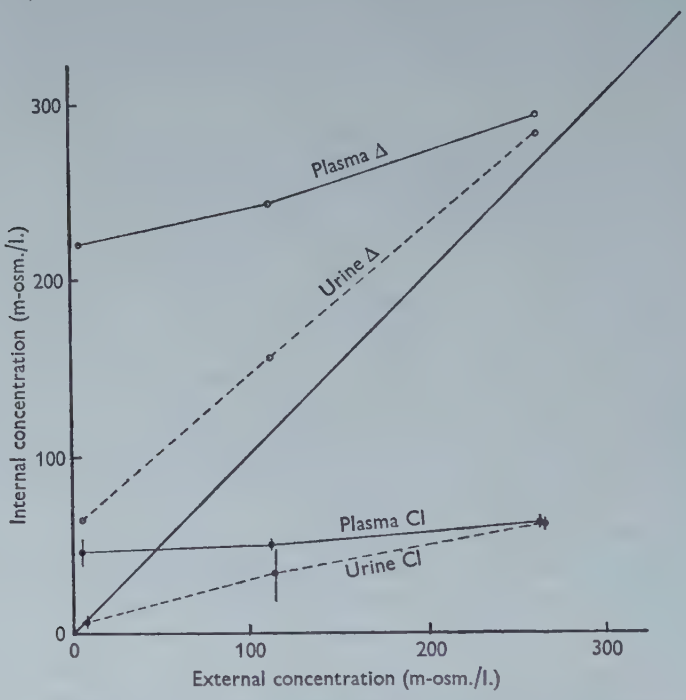

Fig. 9. Relationships between mean plasma and urine osmotic concentration and Cl and environmental osmotic concentration in variously acclimatized groups of *Rana tigerina*. Open circles (○) mean values for osmotic concentration, solid circles (●) mean values for Cl. Solid lines (——) joining points for plasma concentrations, dashed lines (- - -) for urine concentrations. Diagonal solid line, line of equality.

As also in *R. cancrivora*, acclimatization to different media produced no change in evaporation from the skin of *R. tigerina* (Fig. 7). The difference in relative rates of water loss of the two species (Fig. 7) is partially accounted for by differences in surface to volume ratio in frogs of very different size. *R. cancrivora* used weighed 12–20 g. initially, *R. tigerina* weighed 83–143 g. Other contributory differences between the two species are not accounted for.

Experiments testing preference for environmental salinity in groups of *R. tigerina* acclimatized to fresh water and to 25% n.s.w. presented the frogs with a range of choices from fresh water to 20% n.s.w. There were no apparent differences between the two groups, neither showing a marked preference for any specific salinity between fresh water and 15% n.s.w. Fewer than 5% of the observations for each group however, were frogs in 20% n.s.w.

An estimate of the effect of environmental salinity on embryonic development in *R. tigerina* was made as follows: several hundred eggs were stripped from two large

ripe female frogs. These eggs were artificially fertilized with sperm contained in a brei made from excised testes of a ripe male. Groups of about twenty-five eggs were placed in Petri dishes containing 50 ml. of sea water dilutions at 5% intervals from 0 to 40%. The dishes were covered and allowed to stand at room temperature. The eggs were examined after $4\frac{1}{2}$, 18 and 38 hr.

Development of embryos to normal-appearing neurula stages occurred in 18 hr. in 0, 5 and 10% n.s.w., but percentage of successful development declined from about 50% in fresh water to about 23% in 5% n.s.w. and 10% in 10% n.s.w. Virtually all eggs in all three of these concentrations showed some sign of development.

Sea water concentrations of 15% and higher completely prevented normal development beyond what appeared to be early gastrula stages. Some further changes beyond these stages occurred in a small proportion of the eggs in 15 and 20% n.s.w., but the resulting embryos were abnormal. Only 50–75% of all eggs in 15 and 20% n.s.w. showed any signs of development.

In 25% sea water and above no development occurred in any eggs and most eggs were shrunken in appearance.

DISCUSSION

Approximately 300,000,000 years have passed since the appearance of the earliest forms which palaeontologists recognize as amphibians. Present opinion seems to be that two different subgroups of rhipidistid crossopterygians gave rise independently to the amphibian groups today called urodeles and anurans (Jarvik, 1955). Palaeoecological evidence indicates that this transition, for both groups, took place in fresh water environments and almost all amphibian fossils found to date are probably from fresh water areas (Romer, 1945).

The fossil record is far from complete, but it provides evidence that few amphibians have lived in even brackish environments at any time between the Devonian and now (Romer, 1957). Whether or not this situation is due to misinterpretation of palaeoecological evidence, it seems reasonable to view the present possession of considerable salinity tolerance by at least two anurans, *Bufo viridis* and *Rana cancrivora*, as an indication that the present may be either an early stage in an evolutionary development or an only partial success at invading an environment to which amphibia seem unable to adjust. *R. cancrivora* would appear to be an excellent possibility as an evolutionary stem form in either case as it has already entered a rich, widely distributed and unexploited (by amphibians) environment—the mangrove swamps of the tropical world. An inspection of the map given by West (1956) indicates that mangrove swamps along the coasts of continents and major island groups may extend over as much as 100,000 miles.

The physiological mechanism which enables adult *R. cancrivora* to tolerate salinities some three times higher than the highest which other amphibians can tolerate is obviously not a unique evolutionary development among vertebrates. Physiological uraemia is used as a means to several ends in water conservation not only by the elasmobranch fishes, but also by lung fish during estivation (Smith, 1930) and by the African fresh-water frog *Xenopus* during periods of water deprivation (Cragg, 1953, quoted from Underhay & Baldwin, 1955). Even ordinary fresh-water frogs exhibit a tendency in the direction of an increase in plasma nitrogen levels (possibly attributable to urea)

when subjected to elevated salinities (Brunacci, 1914, 1915, 1917*a*, *b*) or desiccation (Przylecki, 1922).

The evolution of an ability to tolerate a uraemia sufficient to be of osmotic significance in brackish and marine environments does, however, appear to be a very difficult feat. Urea concentrations comparable to those measured in the plasma of adult *R. cancrivora* acclimatized to 80% sea water will denature certain enzymes (Elödi & Jecsai, 1960; Riordan, Bier & Nord, 1960) and may affect the oxygen-binding properties of haemoglobin (Rossi-Fanelli, Antonini & Caputo, 1959). The difficulties associated with the osmotically significant use of urea by animals are such that Smith (1936) was prompted to write that a sufficient number of animals had been investigated 'to preclude reasonably the possibility of a physiological uremia [such as that occurring in the elasmobranchs and in the African lungfish]. . . occurring elsewhere in the animal kingdom.'

The continuing physiological uraemia of adult *R. cancrivora* possesses at least one precedent in the elasmobranchs. There may, however, be no precedent, at least among the juvenile stages of oviparous vertebrates, for the osmoregulatory mechanism used by the tadpoles of this frog.

The tadpoles of *R. cancrivora* in our experiments exceeded their parents in salinity tolerance, surviving over the range from fresh water to 120% sea water. If they accomplish this by means of a physiological uraemia, their nitrogen metabolism is very different from the complete ammoniotelism which is a universal property of all premetamorphosis amphibian larvae studied to date (Brown, Brown & Cohen, 1959; Munro, 1953; Underhay & Baldwin, 1955). In view of the physiology of their parents it seems improbable that they exclude salt by mechanisms similar to those of teleost fishes. Their survival may be due to an exceptional tolerance to increased salt concentration in their body fluids. Whatever these tadpoles do, they will amply repay further investigation.

The uraemia of adult *R. cancrivora* probably implies that the skin of this frog is relatively impermeable to urea. This appears not to be the case with ordinary fresh-water frogs (Przylecki, Opienska & Giedroyc, 1922). Impermeability of the integument to urea is not, however, a necessary condition for the maintenance of high levels of uraemia. Fresh-water elasmobranchs, for example, maintain plasma urea concentrations of near 0·1 M in the face of continuing losses to the environment, more than half of which is due to passive diffusion across the gills (Smith, 1931).

The kidneys of *R. cancrivora* apparently conserve some of the urea brought to them by the blood, but appear to be rather inefficient in this regard. Urinary urea levels are always below plasma levels, but are still quite high. This is another resemblance between *R. cancrivora* and the sharks (Smith 1931, 1936).

In fresh-water frogs variation in urea excretion is due to urea secretion and resorption in the renal tubule modifying the urea concentration in the glomerular filtrate (Adolph, 1927; Crane, 1927; Forster, 1954; Love & Lifson, 1958; Schmidt-Nielsen & Forster, 1954). Similar mechanisms may account for the lowered urinary urea values in *R. cancrivora*. However, the relationship between urea concentrations in plasma and urine (Fig. 6) may also be interpreted as the results of passive diffusion into an originally relatively urea-free urine produced by a kidney not using its glomeruli. The demonstration by Richards & Schmidt (1924) and Forster (1942) that many of the

glomeruli in the kidneys of frogs are normally inactive may be relevant. Note should also be made of the fact that histological examination of the kidneys of *R. cancrivora* demonstrates numbers of well-developed glomeruli.

Whatever the details of urea-conserving mechanisms in this frog may be, the basic fact remains that *R. cancrivora* appears to sustain a severe and continuing loss of urea via its urine. An interesting question is the source of all this urea.

The remainder of our data indicate that *R. cancrivora*, having solved the basic problem of water supply in a marine environment, is otherwise very similar to ordinary frogs in fresh water. It has a water-permeable skin, perhaps does not drink external medium, very probably takes up inorganic sodium and chloride from its environment by active transport across its skin, and readily suffers desiccation in air. It is interesting to note that plasma salt concentrations in *R. cancrivora* in 80% n.s.w. are much higher than the plasma salt concentrations which are fatal to *R. tigerina*.

Probably the most striking thing about *R. tigerina* is the fact that, despite its supposed very close phylogenetic relationship to *R. cancrivora*, it appears to be a normal fresh-water frog in every way. Its overall salinity tolerance and osmoregulatory responses to high environmental concentration are virtually identical with those of various of its congeners which have been studied in far distant parts of the world (*R. pipiens:* Adolph, 1927, 1933; Ruibal, 1959; *R. temporaria:* Bertin, 1920; Duval, 1928; Jørgensen, 1954; Overton, 1904; Rey, 1938; *R. esculenta:* Brunacci, 1914, 1915, 1917*a*, *b*, *c*; Przylecki, 1922). The same is true for its response to desiccation (Adolph, 1932, 1933; Durig, 1901; Reichling, 1957; Rey, 1937; 1938, Thorson, 1955, 1956). The decrease in active uptake of sodium by the skin of fresh-water frogs maintained in media more concentrated than fresh water has recently been described by Maetz (1959) for *R. esculenta*.

In closing, note should be made of the possibility that salinity tolerances of amphibia in general may be greater when they are living under natural conditions and not starved in the laboratory. Since urea is an important factor in the tolerance of *R. cancrivora* to high salinities, and since this frog loses appreciable quantities of urea via its urine, well-fed *R. cancrivora* especially may tolerate salinities significantly higher than those just fatal to fasting laboratory animals.

SUMMARY

1. The osmotic and ionic regulatory abilities of adults of the euryhaline crab-eating frog (*Rana cancrivora*) have been studied. Adult frogs tolerated environmental salinities as high as 28‰ at 30° C. Tadpoles of this form tolerated salinities as high as 39‰ at the same temperature.

2. Changes in body weight of frogs following transfers to different environmental salinities indicate both that the skin of this frog is permeable to water and that these animals do not swallow large volumes of external medium, even in high salinities.

3. Above salinities of about 9‰, plasma Δ rises with increasing environmental Δ. Plasma Δ is always higher than environmental Δ. Increases in plasma concentration above fresh-water levels are due partly to increased NaCl concentration (about 40%), partly to increased urea concentration (about 60%). Urea concentrations as high as 0·48 M (2·9%) have been measured.

4. Urinary Δ parallels plasma Δ, but is always lower than plasma Δ. Considerable quantities of urea are lost via the urine, even though urinary urea levels are below plasma levels.

5. Measurements of short-circuit current indicate that active uptake by the skin of inorganic ions continues in *R. cancrivora* acclimatized to high salinities.

6. *R. cancrivora* is no less susceptible to water loss by evaporation from the skin than are other amphibians.

7. In preference experiments *R. cancrivora* chooses salinities below 18‰, but shows no strong preference for a particular salinity.

8. Similar observations on osmoregulatory mechanisms in a close relative of *R. cancrivora*, the tiger frog (*R. tigerina*), show that the latter species is similar to ordinary fresh-water frogs.

9. The striking physiological convergence between *R. cancrivora* and the elasmobranch fishes is discussed, as are various possible implications of our data regarding nitrogen metabolism in tadpoles and kidney function in adult frogs.

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TWO PHASES OF AGEING IN *DROSOPHILA SUBOBSCURA*

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INTRODUCTION

Drosophila, like other poikilotherms, lives for a shorter time at high temperatures and for a longer time at low temperatures. This has commonly been explained by assuming (e.g. Pearl, 1928) that the rates of ageing processes, like those of chemical reactions, are temperature-dependent. Earlier experiments on *D. subobscura* (Maynard Smith, 1958) cast doubts on this interpretation. It was found that if young adult flies were kept at 30° C. for periods equal to about half their expectation of life at that temperature, and were then transferred to 20° C., they died at the same chronological age as did flies kept continuously at 20° C.

These results were incompatible with the assumption that differences in longevity at different temperatures are due to the effects of temperature on the rate of a single ageing process. It was therefore suggested that the causes of ageing at the two temperatures are different. But the results can be explained in another way, as follows. It is supposed that the rate of ageing is, at least approximately, independent of the temperature. Ageing leads to a continuous decline in the ability of individuals to withstand the various factors, internal or external, which may cause death; it is convenient to refer to this as a decline in 'vitality'. The decline continues until the vitality falls below some threshold level appropriate to the temperature at which the animal is living. Once this threshold is passed, the individual starts to die. The level of vitality necessary for survival is higher at higher temperatures, so that differences in longevity at different temperatures are due, not to differences in the rate of ageing, but to differences in the level of vitality necessary for survival at different temperatures.

This hypothesis would explain the earlier results, because individuals transferred from a high to a low temperature before their vitality had fallen to the threshold level appropriate to the higher temperature would be physiologically the same age as flies of the same chronological age kept continuously at the lower temperature. But the hypothesis also predicts that flies which are kept at a low temperature for periods equal to or greater than their expectation of life at some higher temperature, and which are then transferred to the high temperature, should start to die immediately. More generally, for every day an individual is kept at a low temperature, its expectation of life at a high temperature should be reduced by one day.

In this paper experiments are described which confirm this prediction over a wide range of temperatures, although at very low temperatures it appears that the rate of ageing is itself reduced.

METHODS

The flies used were F_1 hybrids between the **B** and **K** inbred lines of *D. subobscura*. They were raised at 20° C. in half-pint milk bottles on a food medium of maize meal, agar and molasses, with 0·5 % dead yeast and two drops of live yeast suspension added. The adults were always kept for 4 days at 20° C. before being transferred to other temperatures. Adults were removed from the culture bottles on the day of emergence and kept subsequently in pairs in 3 by 1 in. diameter vials containing a similar food medium. They were transferred to fresh food vials at regular intervals of 4 days at 20° C. or less, of 2 days at 26° C. and of 1 day at temperatures higher than 26° C.

RESULTS

Table 1 gives the mean longevities of flies kept continuously at various temperatures.

Fig. 1 gives the results of four experiments in which flies were kept initially at a low temperature, and then transferred to a higher temperature until they died. The details are given in Table 2. In no case was there any appreciable mortality at the low temperature before transfer to the higher temperature.

Table 1. *Mean survival times in days at various temperatures*

Temperature (° C.)	Males	Females	
		virgin	mated
3	224	—	—
15	146	> 100*	—
20	83·4	—	58·8
26	35·4	—	—
28·3	—	35·7	—
30	6·4	—	14·0

* A group of twenty virgin females was kept with no deaths for 100 days at 15° C.

Table 2

Exp.	No. of flies given each treatment	Temperature (° C.) Before transfer	Temperature (° C.) After transfer	Age in days at transfer
1	10 ♂♂ / 10 mated ♀♀	20	30	4–48
2	10 ♂♂	20	26	4–48
3	16 virgin ♀♀	15	28·3	4–40
4	16 virgin ♀♀	3	28·3	4–40

If the rate of ageing is in fact independent of the temperature, the curves in Fig. 1 should follow the broken lines, which have been drawn with a slope of $-1{\cdot}0$. The first three experiments agree closely with this prediction during the early part of the life span. Thus in Expt. 2, males transferred to 26° C. when aged 4 days died at a chronological age of 39·4 days, after 35·4 days at 26° C., whereas males transferred from 20 to 26° C., when aged 24 days, died at approximately the same chronological age of 40·2 days, after only 16·2 days at the higher temperature; the longevity of males kept continuously at 20° C. was over 80 days.

But after this initial period, the survival times at high temperatures declined slowly with increasing chronological age at transfer. The explanation for this is probably as follows. Flies whose vitality has fallen below the threshold necessary for survival at a high temperature start to die if transferred to that temperature. But the process of dying may take an appreciable time to reach completion; in fact, it appears to take about 16 days in males kept at 26° C. and about 3 days in males kept at 30° C. Thus the rate of this dying process is highly dependent on temperature.

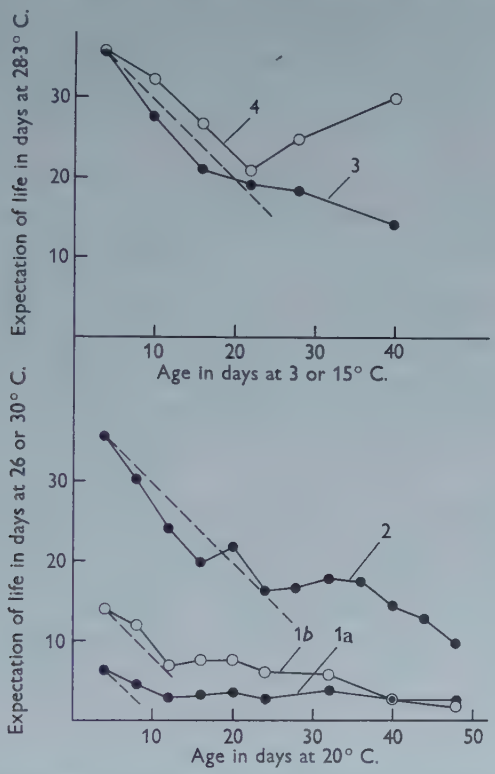

Fig. 1. Mean survival times in days at a high temperature of flies kept previously for varying periods at a lower temperature. 1 *a*, 1 *b*, males and females respectively, transferred from 20 to 30° C.; 2, males transferred from 20 to 26° C.; 3, females transferred from 15 to 28·3° C.; 4, females transferred from 3 to 28·3° C. The broken lines have a slope of −1·0, predicted if the rate of ageing is independent of the temperature.

Expt. 4, in which flies were kept at 3° C. before transfer to 28·3° C., gave an unexpected result; the survival times first decreased and then increased again with increasing chronological age. But it was suspected that this might be a spurious result due to an incorrectly low value of the survival time of the group of females transferred at an age of 20 days. It was therefore decided to repeat this experiment, and at the same time to correct a weakness of Expts. 1 to 3. In these experiments there was no replication of groups given a similar treatment. Consequently there was no method of deciding whether slight changes of temperature or differences between successive batches of food had influenced the results.

In Expt. 5, results of which are given in Table 3, five batches of virgin females were raised in different sets of culture bottles at different times, over a period of about

Table 3. *Expectation of life in days at* 28·8° C. *of virgin females kept for varying periods at* 3 *and* 15° C.

	Chronological age in days at 3° C.				Chronological age in days at 15° C.			
Batch	4	20	40	60	4	20	40	60
a	27·86	—	—	—	27·86	—	—	—
b	—	—	24·85	21·0	—	—	8·20	5·95
c	27·60	22·55	21·65	20·34	27·60	18·35	8·60	8·30
d	28·50	19·75	—	—	28·50	15·56	—	—
e	24·45	18·40	—	—	24·45	9·70	—	—
Mean	27·10	20·23	23·25	20·67	27·10	14·54	8·40	7·12

60 days. Groups of females were transferred to 28·8° C. at chronological ages of 4, 20, 40 and 60 days, having been kept from the age of 4 days either at 15 or at 3° C. Each experimental treatment was replicated at least once, using females from different batches.

Considering first females kept at 3° C., the initial decline in survival time followed by an increase was not repeated, and it is concluded that this earlier result was spurious. The survival times of females kept at 3° C. for from 20 to 60 days were only slightly less than those of control females transferred to 28·8° C. 4 days after emergence. It is concluded that at 3° C. the rate of the ageing process is appreciably slower than it is at higher temperatures.

The results for females kept at 15° C. agree reasonably well with Expts. 1 to 3, although unfortunately there was a rather poor agreement between replicates for females transferred from 15° C. when aged 20 days. From Table 3, the best estimate that can be made of the ratio of the rate of ageing at 28·8° C. to that at 15° C. is $16 \div (27{\cdot}1 - 14{\cdot}54) = 1{\cdot}27$, with a standard error of 0·31. This should be compared to the ratio of over 4:1 for the longevities at the two temperatures. Expts. 1 to 3 suggest a ratio closer to unity, but no estimate can be made of the standard error.

The results of all these experiments are consistent with the hypothesis that the rate of ageing is independent of the temperature from 15 to 30° C. More accurate measurements might show a slightly higher rate at higher temperatures, but the differences in rate are certainly far too small to account for the differences in longevity of flies kept continuously at different temperatures.

REVERSIBILITY OF THE 'DYING' PROCESS

It has been suggested that the lifespan of an individual can be divided into two phases, an irreversible process of ageing whose rate is approximately independent of the temperature, followed by a process of dying in individuals whose vitality has fallen below the threshold appropriate to the temperature at which they are living. The rate of the dying process appears to be highly dependent on temperature. The question arises, are the changes which occur during the dying process at a high temperature reversed if the individual is transferred back to a low temperature?

Four-day-old males survived at 30° C. for a mean of 6·4 days, 8-day-old males for 4·9 days, and males aged from 12 to 64 days for approximately 3 days. This suggests

that freshly emerged males are only just capable of maintaining a steady state at 30° C., and that after a few days their vitality has fallen below the appropriate threshold. The 3-day survival time of older males then represents the duration of the dying phase at 30° C.

Two groups of ten males each were kept for 20 days at 20° C. and then transferred to 30° C. The first group was kept at 30° C. until death, which occurred on the third or fourth day after transfer. The second group was kept alternately for one day at 30° C. and for 3 days at 20° C. They died after total periods at 30° C. of from 9 to 17 days, at chronological ages varying from 53 to 85 days.

This result suggests that the changes which occur during the dying phase and which are ultimately responsible for death can be reversed in individuals transferred to lower temperatures.

CONCLUSIONS

The experiments described above measure the rate at which the expectation of life at a high temperature declines in flies living at a lower temperature. It has been found that over the range 15–30° C., for every day that an individual spends at a low temperature, its expectation of life at a higher temperature is reduced by approximately 1 day. This confirms the hypothesis that the rate of ageing is approximately independent of the temperature over this range, an idea which was first put forward to explain the fact that the exposure of young flies for an appreciable time to a high temperature does not alter their expectation of life at a lower temperature.

A number of rhythmical processes, in *Drosophila* and in other poikilotherms, are known to have periods which are approximately constant over a similar range of temperatures. For example, Pittendrigh (1954) has shown in *D. pseudoobscura* that there is a rhythm of emergence of adults from the pupa, which has a period of approximately 24 hr. over the range 16–26° C., in the absence of any rhythmical environmental stimulus. Unfortunately, there is no reason to suppose that the process of ageing has anything in common with such rhythmical processes, other than its temperature independence.

It is concluded that the life span of a fly can be divided into two phases, referred to as 'ageing' and 'dying'. These may correspond to the processes of 'induction' and 'development' suggested by Neary (1960) from a study of mice. The rate of the ageing process is approximately independent of temperature from 15 to 30° C., but is considerably slower at 3° C. The dying process is initiated when ageing has caused the vitality of the individual to fall below the threshold level necessary if a steady state is to be maintained. Both the level of this threshold and the rate of the dying process are highly dependent on temperature. The damage which occurs during the dying process at a high temperature can be repaired in flies transferred to a lower temperature.

SUMMARY

1. Male and female *D. subobscura* were kept for varying periods at low temperatures (3–20° C.) and then transferred to a higher temperature (26–30° C.) and kept there until they died.

2. It was found that during the early part of the life span, over the range 15–30° C., every day spent at a low temperature reduced the expectation of life at a higher

temperature by approximately 1 day. Later, when the expectation of life at the higher temperature had fallen to about half its initial value, little further change in this expectation occurred with increasing age at a lower temperature.

3. It is concluded that the life span can be divided into two phases, (i) an irreversible 'ageing' process whose rate is approximately independent of temperature from 15 to 30° C., but which is slower at 3° C., and (ii) a 'dying' process which is initiated when ageing has proceeded to a stage at which the individual is no longer capable of maintaining a steady state at the temperature at which it is living, although the same individual would be capable of maintaining a steady state at some lower temperature.

4. The rate of the dying process is highly dependent on temperature, and it can be reversed in flies transferred to lower temperatures.

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THE SWIMMING RESPONSE AND ITS PACEMAKER SYSTEM IN THE ANEMONE *STOMPHIA COCCINEA*

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The swimming behaviour of the anemone *Stomphia coccinea* Müller has been described by several authors (Stephenson, 1935; Yentsch & Pierce, 1955; Sund, 1958; Wilson, 1959; Hoyle, 1960; Robson, 1961). Specimens from the region of Puget Sound and the San Juan Islands respond to contact with certain starfish or to suitable electrical stimulation with a characteristic sequence of activity. They retract, re-expand, detach from the substratum, and display a series of abrupt bending movements which may continue for several minutes. An inert recovery period is followed by re-attachment to the substratum. The only species so far known to provoke this response are the starfish *Hippasteria spinosa* Verrill and *Dermasterias imbricata* Grube. The world distribution of the genus *Hippasteria* overlaps to some extent that of *Stomphia*, which is a widespread North European–Arctic form, but *Dermasterias* is strictly indigenous to the north-west American coast. Neither starfish seems to affect the anemone in any other way, and the significance of this particular response is difficult to understand.

It is thus of especial interest that K. W. Ockelmann* has now found that *Stomphia coccinea* from the Öresund–Kattegat region will swim in response not only to *Hippasteria phrygiana* Parelius but also to *Aeolidia papillosa* L. Since the nudibranch will feed on *Stomphia* and occurs in the same habitat, the swimming behaviour can be regarded as an escape reaction to predators which is also evoked by certain starfish. This interpretation seems valid as *Aeolidia* has been reported from the Pacific (Abbott, 1954; but see Pruvot-Fol, 1954). A preliminary comparison of the responses to *Aeolidia* and to *Hippasteria* suggests, as will be seen, that the sensory pathways involved may be different in each case. Both stimuli are able to excite a pacemaker system which is active during swimming.

MATERIAL AND METHODS

Most of the present observations were made at the Marine Biological Laboratory, Helsingør, Denmark. Material was obtained from Knähaken (just south of Helsingborg) by dredging at 27 m. As in Puget Sound, *Stomphia coccinea* commonly occurs on shells of *Modiolus modiolus*, and both habitats appear to be similar in other respects (see Sund, 1958; Brattström, 1941). One specimen of the rare *Hippasteria phrygiana* and five *Aeolidia papillosa* were also available. These and some of the anemones were kept in circulating sea water at 12° C. Other anemones were kept in bowls at 10° C., as this temperature approximated better to that of the habitat (annual range 5–12° C.).

* Unpublished observations.

Living specimens of *Stomphia coccinea* from Knähaken closely resemble those from Puget Sound. Dr Cadet Hand has kindly compared fixed specimens from both localities and finds them to be identical (personal communication).

Details of methods not given below have already been described (Robson, 1961).

THE RESPONSE TO *AEOLIDIA PAPILLOSA*

The specificity of response to the starfish *Hippasteria phrygiana* and the lack of effect of *Crossaster papposus* and *Solaster endeca* from the same habitat are easily demonstrated, and confirm Sund's observations on Puget Sound material (1958).

Table 1

	Swimming response				
	+	partial	−	Trials	No. of anemones tested
Hippasteria phrygiana	8	2	0	10	10
Crossaster papposus	0	0	10	10	10
Solaster endeca	0	0	10	10	10

Tests were carried out in fresh sea water at 10° C. In each case the aboral surface of the starfish was in contact with the crown of tentacles for 60 sec., although with *Hippasteria* a response is usually seen after about 10 sec. Fig. 1 A shows an activity record of swimming evoked by *Hippasteria*.

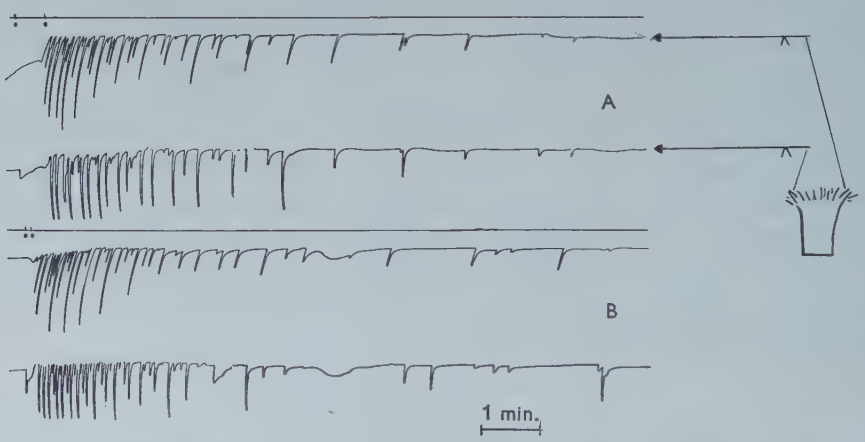

Fig. 1. Swimming responses of a fixed anemone recorded from threads attached to opposite points of the disk. A. *Aeolidia* in contact with the column. Dots on the upper tracing mark duration of stimulus. B. *Hippasteria* in contact with tentacles. Dots again mark duration of stimulus.

Swimming due to *Aeolidia papillosa* follows the same pattern (Fig. 1 B), although as will be seen the initial response is different. A nudibranch about to feed on one of the anemones approaches it at ground level and the foot makes contact with the lower part of the column. The *Aeolidia* usually secures a hold by biting deeply into the anemone, neatly puncturing the lower part of the column, whereupon the anemone retracts vigorously. It may then expand and swim, often shaking off the mollusc.

Frequently, however, the anemone swims before the nudibranch has bitten it, showing that contact between the foot and the column is an effective stimulus. This is the only explanation for the rapid response of some totally retracted anemones after an *Aeolidia* had crawled over them for less than half a minute.

An interesting feature of this response is that it does not usually involve the anemone's tentacles or sphincter. With *Hippasteria* and *Dermasterias* a contraction of sphincter and retractor muscles precedes swimming, but *Aeolidia* often produces no such retraction. The cerata do not evoke a response, nor does contact between any part of the mollusc and the anemone's tentacles. The nudibranch in fact withdraws if it is stung by nematocysts. All observations suggest that a substance produced particularly by the anterior part of the foot (i.e. in the region of the slime groove) causes swimming when applied to the column.

The substance produced by *Aeolidia* is chemically specific. *Aeolidiella glauca* L., a similar nudibranch from the same habitat, does not cause swimming in *Stomphia*, nor do *Armina lovéni* Bergh or the large *Tritonia hombergi* Cuvier, as may be seen from the following tests.

Table 2

	Swimming response				
	+	partial	−	Trials	No. of anemones tested
Aeolidia papillosa	76	40	30	146	53
Aeolidiella glauca	0	?3	30	33	32
Armina lovéni	0	0	10	10	10
Tritonia hombergi	0	?3	7	10	10

Tests were carried out in fresh sea water at 10° C. A negative response was recorded only after the mollusc had crawled over the column of an anemone or had been held in position for 2 min. or longer.

These results accord with the feeding habits of the nudibranchs. *Aeolidiella glauca* appears to feed mostly on *Sagartia* sp., whereas *Aeolidia papillosa* appears to prefer *Stomphia* to *Metridium* or *Tealia* and rarely takes *Sagartia*, at least in the Danish Sound (K. W. Ockelmann, unpublished observations, and personal observation; see also Stehouver, 1952; Braams & Geelen, 1953; Miller, 1961). *Tritonia hombergi* normally browses on *Alcyonium digitatum* (Alder & Hancock, 1845).

Simple aqueous extracts of the chemical substances produced by *Hippasteria* and *Dermasterias* will make *Stomphia* swim even in the absence of a mechanical stimulus (Ward, 1958; Robson, 1961), and a similar extract was therefore prepared from one of the *Aeolidia*. The head and foot, cerata, and remaining part of the body were treated separately. The tissues were fragmented with a razor blade, and ground by hand with 3–4 ml. sea water. Centrifuging at 5000 r.p.m. for 10 min. then produced a slightly turbid supernatant fluid which was used for testing. Preparation took less than an hour and the extracts were kept over ice. They were tested on anemones in dishes of clean sea water at 10° C., the extract being delivered 1–2 cm. above the expanded disk and tentacles with a pipette. Results are summarized in Table 3.

It may be concluded that *Aeolidia* produces a chemical substance causing swimming, but that it is probably not the same as the substance found in *Hippasteria* and *Dermasterias*. From Ward's account (1958) starfish extract is inactivated by boiling and

dialysis, which was not the case here. Starfish extract also produces a rapid sphincter contraction (Robson, 1961), which was conspicuously absent in most of the tests with *Aeolidia* or the extracts. As will be seen, it is probably a case of two substances acting at different receptor sites in the anemone.

Table 3

Aeolidia extract	Test	No. of drops	Response
A. Foot and head	Fresh extract	5 or 10	Vigorous swimming
	After 15 min. over boiling water	5	Vigorous swimming
	Boiled over flame for 2 min.	5	Vigorous swimming
	Dialysed 26 hr. against running sea water at 12° C.	10	Fairly vigorous response
	Control kept 26 hr. at 4° C.	10	Vigorous swimming
B. Cerata	Fresh extract	40	Disk swells, mouth opens, swimming response absent
	Fresh extract B followed by extract A (control)	15 10	Negative response as above but control extract produces vigorous swimming
C. Remaining tissues	Fresh extract	5 or 10	Partial or full swimming response but less vigorous than extract A

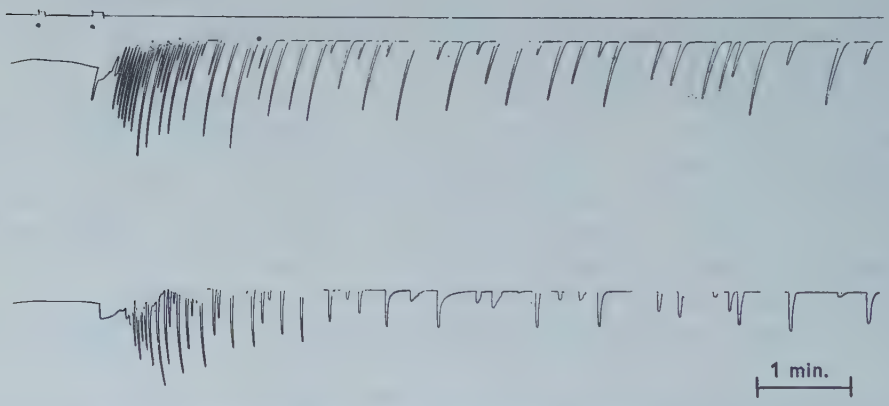

Fig. 2. Swimming response of a fixed anemone to extract of *Aeolidia* head and foot (p. 687). Dots on the upper tracing mark the addition of five and then seven drops of fresh extract near the oral disk.

Fig. 2 gives some idea of the swimming movements produced by the extract of *Aeolidia* head and foot. It is part of a kymograph tracing in which the activity of a fixed anemone is recorded by means of two threads at opposite points on the disk (cf. Fig. 1). Very similar records are obtained with *Dermasterias* extract (Robson, 1961), but a direct comparison of starfish and nudibranch extracts has not yet been made. If, however, an anemone is stimulated several times with *Aeolidia* until finally no

response occurs on contact with the mollusc, stimulating the tentacles with *Hippasteria* may still evoke quite lively swimming movements.

This suggests not only that the response to repetitive stimulation with *Aeolidia* falls away because of sensory adaptation or fatigue, but also that the nudibranch and starfish chemicals act on different sensory regions of the anemone. Since *Aeolidia* appears to stimulate the column directly, a curious anatomical problem arises. Sense cells in the ectoderm must transmit excitation to the endodermal elements which carry out the swimming response. But in sections of *Stomphia* no evidence that the processes of sense cells or nerve cells cross the mesogloea of the column from ectoderm to endoderm can be found. It remains possible that ectodermal sense cells in the column excite endodermal elements via the pedal disk. The parieto-basilar muscle fibres are unusual in penetrating the mesogloea of the pedal disk as far as the ectodermal epithelium, and could thus provide a possible anatomical pathway for the processes of sense cells or nerve cells linking ectoderm and endoderm (Robson, 1961).

Histological study of *Aeolidia* has not so far elucidated the nature or source of the stimulating substance. The anterior part of the foot appears to be more effective than the rest, and the only special gland in this region is the anterior pedal gland, which discharges along the pedal groove. Similar subdermal gland cells are also distributed over the whole pedal surface. These cells all appear to produce a continuous secretion containing mucopolysaccharide, and until further evidence is obtained, they are perhaps the most likely elements to be concerned. There are, however, at least three other types of gland cell in the pedal epithelium. In the material available it is difficult to distinguish their different phases of secretion, and the problem awaits further investigation (see, however, Campion (1961)).

THE NATURE OF SWIMMING ACTIVITY

Once the swimming activity evoked by starfish, *Aeolidia* or electrical stimulation is set up (Yentsch & Pierce, 1955; Wilson, 1959; Hoyle, 1960), it is independent of further stimulation and may continue for several minutes. The column elongates, owing to contraction of the circular muscle, and undergoes a series of stacatto bending movements brought about by contractions of the parieto-basilar muscles. It was suggested in a previous paper (Robson, 1961) that the synergic activity of these two sets of muscles is maintained locally on the column, and that parieto-basilar contractions were due more probably to a pacemaker system than to sensory or nervous reflexes. Evidence that a pacemaker system is present has now been obtained by considering the orientation of bending movements in relation to the position of the initial stimulus, and their resumption after swimming has been interrupted.

When the orientation of bending movements is observed in a swimming anemone, the alternation of parieto-basilar contractions at opposite radii is usually seen to be modified by a slow migration of the sites of excitation round the column. This slow rotation (about once a minute) does not seem to be an active process, since it can reverse in direction and may not be established until after some initial oscillation has taken place. It indicates, however, the functional equivalence of all the parieto-basilar elements in the column (Robson, 1961). It is therefore not surprising that the first

movements are orientated according to the position of the initial sensory stimulus. Detachment of the pedal disk from the substratum usually proceeds from the stimulated radius. The first parieto-basilar contraction is usually also at this radius, and thus depends on which tentacles are in contact with a starfish, or which region of the column is stimulated by *Aeolidia*. A strictly local parieto-basilar contraction also results from gently prodding the column in both *Stomphia* and *Tealia* (cf. the rapid parietal response in *Bunodactis* described by Pantin & Vianna Dias (1952*a*)), and it is thus the prolonged repetition of bending movements at sites which apparently need be little related to the initial stimulus that is unusual in *Stomphia*.

The pacemaker system

A swimming anemone retracts to a sharp prod, but as the sphincter and retractor muscles relax, swimming is resumed (Wilson, 1959). It is known that halves of anemones transected during swimming continue to swim, and that both vertically divided portions and horizontally divided portions of anemones can respond to stimulation with *Dermasterias* (Wilson, 1959; Robson, 1961). When a vigorously swimming anemone is cut into two vertical halves, the halves presently relax, elongate once more and both proceed with the interrupted series of parieto-basilar contractions. If this operation is done while carefully observing the bending sequence up to the time of cutting, it is found that the first parieto-basilar contraction seen in the separated halves is always at the site that would have been expected were the anemone still intact. This makes it clear that the anemone must possess some kind of pacemaker system, and that swimming movements do not arise as a series of sensory reflexes.

The pacemaker system has been localized in an equally crude manner by cutting swimming anemones horizontally. If the transection is made one-third from the base of the column, only the upper portion resumes swimming. If the cut is one-third from the top of the column, the basal portion only resumes swimming. The pacemaker system is thus in the middle third of the column, and may be totally inactivated if a horizontal cut happens to hit on the right latitude about halfway up the column. It may thus be visualized as a circular zone of nervous activity in the column which excites the parieto-basilar muscles locally. This is shown also by a transection oblique to the main axis of the column: the two portions resume parieto-basilar contractions only in those sectors containing the pacemaker ring.

The pacemaker system can be excited either by local sensory stimuli anywhere on the crown (starfish) or on the lower column (*Aeolidia*), or by suitable electrical stimulation. Hoyle (1960) has noted that the position of electrodes on the column is important, and present observations confirm that the pacemaker system seems to be excited only by shocks delivered in its vicinity, Hoyle found that the local parieto-basilar contractions are produced by single electric shocks. It is thus simplest to suppose that during swimming each parieto-basilar contraction is due to one impulse in the pacemaker system. Electrical stimulation sometimes produces swimming movements without affecting the circular muscle. Thus the customary elongation of the anemone during swimming probably does not depend on the pacemaker.

Inspection of swimming records has failed to reveal any clear-cut patterns of rhythm beyond the exponential decline of parieto-basilar contraction frequency in any

response (e.g. Figs. 1, 2). The pacemaker ring fires off at numerous sites during swimming. These are all potentially labile in position, and usually do not fire regularly. The activity of the system is nevertheless integrated as a whole, and it would seem that excitation in any part of the ring to some extent influences the rest. This is seen particularly in the swimming response of isolated vertical halves. In such preparations sites of contraction do not migrate as readily as in intact anemones (p. 689), and it is usual for contractions to take place at a few radii only. Timed records from preparations show that parieto-basilar contractions at 2, 3 or 4 radii occur in fairly random order, but sometimes a statistically non-random series appears which can be interpreted as rhythmical activity at those focal points. If a recurring interval between contractions at two different radii becomes short, temporary synchronization may develop. This points to the integration of activity within the pacemaker ring since the rhythm of two or more centres is modified.

It has been noted previously that in intact anemones two contractions following at a short interval tend to occur at diametrically opposite radii (Robson, 1961). If an impulse at radius *X* were followed by a period of decreased probability of excitation, the most probable radius of firing during this period could reasonably be at the diametrically opposite radius *Y*. In fact the rapid sequence at the beginning of a response often consists of a sustained *XY* series, which only later includes or moves to other radii. If recent impulses can influence the position and timing of subsequent ones, both positive and negative effects may be expected. Other parts of the nervous system can also modify pacemaker activity since, for example, swimming can be interrupted or slowed down by stimulation of the sphincter-retractor system (p. 690). It seems that the appearance of leading radii in certain sequences may derive from particularly excitable sectors of the pacemaker ring, or else from the asymmetrical position of the initial stimulus.

It is to be hoped that future histological work may reveal nerve cells in the column corresponding to the position of the pacemaker ring. In the meantime it appears that swimming in *Stomphia* has several features in common with rhythmical activity of nervous origin in certain other invertebrates.

DISCUSSION

In most other sea anemones any rhythmical activity is usually far too slow to have originated in the kind of pacemaker system present in *Stomphia* (Batham & Pantin, 1950*a*, *b*; Ewer, 1960; Robson, 1961). The comparably rapid movements of *Boloceroides* and *Gonactinia* which are reported to swim have not yet been studied. There are, however, similarities in the better known locomotory rhythm of medusae, which originates in the marginal ganglia. The eight or more ganglia are connected by a nerve ring (a specialized through-conducting region of the subumbrellar nerve-net), and give rise to rhythmical pulsation of the bell. Isolated portions of this system each beat at different rates, and more slowly than the intact medusa (Horstmann, 1934*a*, *b*). They normally give a much more regular and prolonged rhythm than is ever seen in a swimming *Stomphia*. But, as in *Stomphia*, extrinsic sensory or nervous excitation can either accelerate or inhibit the pacemakers. Although the pacemaker ring is much more loosely organized in *Stomphia* than in medusae, both exhibit similar properties

attributable to the cnidarian nerve-net (see Horridge, 1955*a*, *b*; 1956*a*, *b*; 1959; Pantin & Vianna Dias, 1952*b*).

Swimming activity in *Stomphia* also has something in common with the long after-discharge of luminescent flashes produced by the sea-pansy *Renilla* when stimulated vigorously (Nicol, 1955*a*, *b*) and by *Leioptilus* (Davenport & Nicol, 1955). Strong mechanical stimuli produce a train of luminescent waves across the colony which arise rhythmically from the stimulated area. The nerve-net in which they are propagated is diffuse, and shows non-polarized through-conduction. Prolonged electrical stimuli may raise the general level of excitation until numerous local pacemakers are established, each setting up waves of scintillation for anything up to 30 min. Two other observations on sea-pens are of interest. In *Pennatula*, if waves of luminescence travelling in opposite directions meet, they tend to summate but do not pass each other at the junction (Panceri, 1872), suggesting that excitation arriving from more than one source may produce inhibition in a region of the nerve-net. This agrees with observations on medusae (Horridge, 1955*b*, 1956*a*) and probably occurs in *Stomphia*. Secondly, although at low frequency several shocks are required to initiate luminescence in *Renilla*, the effect of the first few may sometimes be seen as small flashes at points scattered all over the colony. Nicol (1955*b*, 1960) interprets this as the random distribution of photocytes with particularly low thresholds. It is evidence that in this system some of the effector cells at least are hyperexcitable. In considering the nervous organization of temporary pacemakers, it is difficult not to assume that some comparable heterogeneity exists.

Nicol (1953, 1954) has also examined luminescence in polynoid worms. In isolated scales one electric shock gives rise to a rhythmical train of flashes which depends on the presence of the elytral ganglion. When the ganglion is absent, one shock produces only one flash. The temporary rhythm must thus arise from interaction between excited nerve cells. A similar conclusion has been reached by Horridge (1957) in considering the behaviour of certain alcyonacean colonies. The response of a colony of *Sarcophyton* zooids to electric shocks, although not rhythmical, is cumulative with respect to frequency and number of stimuli. Horridge explains this by suggesting that the excitation interacts with itself until a point is reached at which the activity is self-maintaining. Although the idea refers here to the establishment of through-conduction, it appears to hold in general also for any rhythmical system set in motion by electrical stimulation. Another colony, *Heteroxenia*, shows continuous rhythmical activity of all the polyps (Horridge, 1956*c*), but since no two polyps are co-ordinated, it would seem here as though each had its own pacemaker.

There are other cases in which a pacemaker is known to reside in a relatively small number of nerve cells. Rhythmical activity of the pharynx in *Arenicola* arises from a pacemaker in the oesophageal wall, portions of which are radially equivalent as in *Stomphia* (Wells, 1937, 1950). Histological study has revealed a nerve plexus in this region (Whitear, 1953), which must therefore be the seat of the rhythm. By contrast, the rhythm of the heart in higher Crustacea originates in a few cells of the cardiac ganglion (Maynard, 1960). Despite their restricted localization in this case, it has been shown that pacemaker cells at times fire independently, and at times interact. This is no different from any other system showing temporary or sustained 'spontaneous' nervous activity, including the pacemaker ring in *Stomphia*.

Although many other examples could be given, those discussed above show that the swimming response of *Stomphia* could plausibly depend on the temporary excitation of a pacemaker ring, whose activity would have much in common with rhythms known in other cases to arise in a nerve-net or simple ganglion.

SUMMARY

1. The swimming reaction of the anemone *Stomphia coccinea* to *Hippasteria phrygiana* from Danish waters is identical with that of North American Pacific coast anemones to the starfish *H. spinosa* and *Dermasterias imbricata*.

2. The swimming reaction is also evoked by the nudibranch *Aeolidia papillosa*, which feeds on the anemone (K. W. Ockelmann, unpublished observations). The foot of the mollusc secretes a specific chemical which is not the same as that produced by the starfish, and probably acts at different receptor sites in the anemone. The swimming response is interpreted as an escape reaction to predators which is also evoked by certain starfish.

3. Cutting experiments show that parieto-basilar contractions during swimming arise from a pacemaker ring about halfway up the column, and not from sensory or nervous reflexes. Parts of the system are radially equivalent. Once excited by effective sensory or electrical stimuli, the pacemaker shows properties common to other sources of rhythmical nervous activity. Sites of firing are labile but influence others, and the activity of the intact system is correspondingly integrated.

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